



ROLE OF THE REDOX RESPONSIVE TRANSCRIPTION FACTOR, NRF2 IN IMMUNE CELL FUNCTION

Thesis submitted in accordance with the requirements of the University of
Liverpool for the degree of Doctor in Philosophy
by

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DECLARATION

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part for any degree or other qualification.

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This research was carried out in the MRC Centre for Drug Safety Science,
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For Bridie and Ham

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ABSTRACT

Dendritic cells (DCs) are potent innate antigen presenting cells which are able to sense and engulf pathogens from sites of infection, which are then processed and presented to adaptive T lymphocytes in secondary lymphoid organs. Therefore, they are critical for the initiation and modulation of primary antigen-specific adaptive immune responses. They also play a critical role in the maintenance of T cell tolerance. T cells play vital roles in mediating both cellular and humoral-specific adaptive immune responses. There are various types of T cells present within the immune system including the cytotoxic CD8 T cells and T helper CD4 cells. CD4 T cells can be further subdivided into various subtypes examples of which include T helper 1 cells (Th1), Th2, Th17 and T regulatory cells. Each subset has its own distinctive function, transcriptional regulation and effector cytokine profile.

It is established that appropriate DC and T cell immune function is highly dependent on their intracellular redox status. Cellular redox homeostasis is maintained through a balance between oxidising agents e.g. reactive oxygen species (ROS) and anti-oxidant or reducing agents e.g. glutathione (GSH). Excessive ROS production resulting in oxidative stress is extremely deleterious to the cell and if left unimpeded can result in cell necrosis, tissue damage and the onset of disease. As a result, mammalian cells have evolved an inducible adaptive defence system which provides protection against such oxidative or chemical insult. The functionality of this cellular defence system is principally governed by the activity of the redox-sensitive transcription factor Nrf2. In response to oxidative stress, Nrf2 induces the transcription of a battery of cytoprotective and antioxidant genes involved in GSH synthesis, detoxification of xenobiotics and their reactive metabolites and the maintenance of cellular redox homeostasis. It is now emerging that Nrf2 plays a pivotal role in immunity. However, its precise role in DC and T cell function is unclear.

Using immature bone marrow-derived DCs (iDCs) from Nrf2^{+/+} and Nrf2^{-/-} mice, the work presented in this thesis demonstrates that Nrf2 deficiency in iDCs resulted in lowered GSH levels, enhanced iDCs co-stimulatory receptor expression, impaired endocytic and phagocytic capacity, and increased iDC-mediated antigen-specific CD8 T cell stimulatory capacity in response to both an antigenic and self-peptide. Furthermore, artificially lowering GSH levels in the iDCs did not recapitulate the Nrf2 deficient iDC phenotype. Moreover, Nrf2^{-/-} DCs exhibited an enhanced capacity to present cell-associated peptide antigens to antigen-specific CD8 T cells, resulting in increased CD8 T cell effector function. Loss of Nrf2 in LPS-stimulated DCs results in a lowered Th1 cytokine profile. These results have implications for Nrf2 in DC-mediated CD8 T-cell immunity, peripheral CD8 T cell tolerance and CD4 effector differentiation.

The role of Nrf2 in T cell function is poorly understood. Using thymocytes, splenocytes and lymph-node derived T cells from Nrf2^{+/+} and Nrf2^{-/-} mice, we demonstrate that loss of Nrf2 did not affect the development of CD4⁺CD25⁺ natural occurring Treg, mature CD4 and CD8 T cell populations within the thymus.

Furthermore, Nrf2 deficiency did not alter the composition of CD4 and CD8 T cell populations within secondary lymphoid organs. It was observed that splenic Nrf2^{-/-} naïve T cells exhibited enhanced ROS generation, accompanied by low level increases in T cell activation markers. However, the marginal augmentation of Nrf2^{-/-} naïve T cell activation status did not result in increased T cell receptor (TCR)-triggered T cell proliferation. In contrast, Nrf2 deficient effector T cells exhibited enhanced TCR/CD3-triggered proliferation, associated with increased Th1 and decreased Th2 effector function. Importantly, we demonstrated that Nrf2^{-/-} effector T cells secreted increased levels of IL-17A and IL-22, a signature cytokine profile indicative of the more recently identified Th17 cell lineage. This was also observed under Th17 polarising conditions, further suggesting that loss of Nrf2 predisposes effector Th17 development. The implications of the latter findings are significant given the pivotal role that Th17 cells play in the pathogenesis of a variety of autoimmune diseases including multiple sclerosis (MS) and Systemic lupus erythematosus (SLE).

Nrf2 plays a critical role in the detoxification of xenobiotics in the liver, which as the primary drug-metabolising organ, is subjected to an array of xenobiotics and their respective metabolites. Individuals vary in their responses to xenobiotic exposure from adaptation to severe adverse drug reactions. However, it is unknown whether this human disparity in drug response is a consequence of inter-individual variation in the Nrf2 adaptive defence system to xenobiotic stress. In light of this, we aimed to firstly investigate whether variation in the Nrf2 adaptive system was present within individuals in response to a chemical inducer of Nrf2, CDDO-Me. To address this issue, basal and induced Nrf2 protein levels and downstream NQO1 expression were measured in activated human T cell blasts, in response to increasing concentrations of CDDO-Me. Examination of various donor-derived T cells, demonstrated that humans vary in their Nrf2 response to CDDO-Me, with respect to nuclear Nrf2 and NQO1 mRNA expression. Therefore we concluded that inter-individual variation does exist in the human's Nrf2 adaptive system in response to a known Nrf2 probe.

Overall, the experimental results obtained in this PhD programme have provided detail on the role of Nrf2 in immune cells and highlights the potential for therapeutically targeting Nrf2 in immune-mediated disease.

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PUBLICATIONS

Papers

Laith M A Al-Huseini, Han Xian Aw Yeang, Swaminathan Sethu, Alumeed Naif, **Junnat M Hamdam**, Laiche Dijouri, Neil Kitteringham, B Kevin Park, Christopher E Goldring and Jean G Sathish. Nuclear factor-erythroid2 (NF-E2) p45-related factor-2 (Nrf2) modulates dendritic cell immune function through regulation of p38-CREB/ATF1 signalling (2013). *Journal of Biological Chemistry*. 2;288(31):22281-8.

Han Xian Aw Yeang*, **Junnat M Hamdam***, Laith M A Al-Huseini, Swaminathan Sethu, Laiche Djouhri, Joanne Walsh, Neil Kitteringham, B Kevin Park, Christopher E Goldring and Jean G Sathish. Loss of the transcription factor nuclear factor-erythroid2 (NF-E2) p45-related factor-2 (Nrf2) leads to dysregulation of immune functions, redox homeostasis and intracellular signalling in dendritic cells (2012). *Journal of Biological Chemistry*. 287(13):10556-64 (*equal contributors)

Reviews

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ABBREVIATIONS

μ;	micro
2-ME;	2-Mercaptoethanol
Ag;	antigen
ADR;	adverse drug reaction
AHR;	aryl hydrocarbon receptor
AKR;	aldo-keto reductase
ANOVA;	analysis of variance
AP-1;	activator protein 1
AP-3;	adaptor protein 3
APC;	antigen presenting cell
APL;	altered peptide ligand
ARE;	antioxidant response element
ARNT;	AHR nuclear translocator
ATP;	adenosine triphosphate
BDCA;	blood dendritic cell antigen
BCR;	B-cell receptor
BCL6;	B cell lymphoma-6
BHA;	butylated hydroxyanisole
BM;	bone marrow
BMDc;	bone marrow-derived dendritic cell
BSA;	bovine serum albumin
BTAF;	B cell-Activating Transcription Factor
BTB;	bric-a-brac/tram-track/broad complex
bZip;	basic leucine zipper
C;	Celsius
Ca ²⁺ ;	calcium
CaN;	calcineurin
cAMP;	cyclic adenosine monophosphate
CBP;	CREB-binding protein
CCR;	chemokine (C-C motif) receptor
CCL;	chemokine (C-C motif) ligand
CD;	cell surface molecules expressed on various cell types
cDC;	conventional DC
CDDO;	2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid
CDDO-Im;	CDDO imidazolidine
CDDO-Me;	CDDO methyl ester also known as Bardoxolone methyl
cDNA;	complementary DNA
CER;	cytoplasmic extraction reagent
CFSE;	carboxy fluorescein succinimidyl ester
CGD;	chronic granulomatous disease
CLEC9A;	C-type lectin domain family 9A also known as DNGR-1
CLIP;	class II-associated invariant chain peptide
CLP;	common lymphoid progenitor

CLR;	C-type lectin receptor
CM;	complete media
CMP;	common myeloid progenitor
CNC;	cap 'n' collar
CO ₂ ;	carbon dioxide
CON-A;	Concanavalin A
COPD;	Chronic obstructive pulmonary disease
CpG;	C-phosphate-G
CREB;	cAMP responsive element binding protein
C region;	constant region
Csk;	C-terminal Src kinase
CT;	cross threshold
CTL;	cytotoxic T lymphocyte
CTLA-4;	cytotoxic T lymphocyte antigen 4
CUL3;	Cullin 3
CXCR;	chemokine (C-X-C motif) receptor
CYP450;	cytochrome P450
Cys;	cysteine
Da;	Dalton
DAG;	1,2-diacylglycerol
DC;	dendritic cell
DC-SIGN;	DC-Specific Intercellular adhesion molecule-3-Grabbing Non integrin
DEC-205;	C-type lectin receptor (205 kDa) also known as CD205
DGR;	double glycine repeat
DHE;	dihydroethidium
dH ₂ O;	distilled H ₂ O
DILI;	drug-induced liver injury
DL;	delta-like
DEM;	diethyl maleate
DMSO;	dimethyl sulphoxide
DN;	double negative (CD4 ⁻ CD8 ⁻)
DNA;	deoxyribonucleic acid
DNCB;	2,4-dinitrochlorobenzene
DNTB;	5,5'-dithio-bis(2-nitrobenzoic acid)
dNTP;	deoxyribonucleotide triphosphate
DP;	double positive (CD4 ⁺ CD8 ⁺)
dsRNA;	double stranded RNA
DTNB;	5,5'-dithiobis(2-nitrobenzoic acid)
EAE;	experimental autoimmune encephalomyelitis
E. coli;	Escherichia coli
EC ₅₀ ;	half maximal effective concentration of a drug
ECH;	erythroid cell-derived protein with CNC homology
ECL;	enhanced chemiluminescence
EDTA;	ethylenediaminetetraacetic acid

ELISA;	Enzyme-linked immunosorbent assay
EOMES;	eomesodermin
ER;	endoplasmic reticulum
ERK;	extracellular signal-regulated kinase
EtOH;	ethanol
FACS;	fluorescence activated cell sorting
FasL;	Fas ligand
FBS;	fetal bovine serum
Fc;	Ig-constant region
FcR;	Fc receptor
FCS;	fetal calf serum
FITC;	fluorescein isothiocyanate
FLT3;	Fms-like receptor tyrosine kinase 3
FLT3L;	FLT3 ligand
FoxP3;	forkhead box P3
Gads;	Grb2 related adaptor protein downstream of Shc
GAPDH;	Glyceraldehyde 3-phosphate dehydrogenase
GATA-3;	GATA binding protein-3
GCL;	γ -glutamylcysteine ligase
GCLC;	GCL, catalytic subunit
GCLM;	GCL, regulatory subunit
GDP;	guanosine diphosphate
GEF;	guanine nucleotide exchange factor
Gfi-1;	growth factor independent-1
GI;	gastrointestinal
GM-CSF;	granulocyte-macrophage colony-stimulating factor
GPI;	glycosylphosphatidyl inositol
GPX;	GSH peroxidase
GS;	GSH synthetase
GSH;	glutathione
GSR;	GSH reductase
GSSG;	oxidised GSH or glutathione disulphide
GST;	GSH S-transferase
GVHD;	Graft-versus-host disease
H ₂ O;	water
H ₂ O ₂ ;	hydrogen peroxide
H ₂ SO ₄ ;	sulphuric acid
HAT;	histone acetyltransferase
HBSS;	Hanks balanced salt solution
HBV;	Hepatitis B virus
HCl;	hydrochloric acid
HCV;	Hepatitis C virus
HDAC;	histone deacetylase
HEPES;	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HES-1;	hairy and enhancer of split-1
HIF-1;	hypoxia-inducible factor 1
HIV;	human immunodeficiency virus
HLA;	Human leukocyte antigen
HMGB1;	High-Mobility Group Box 1 Protein
HO-1;	heme oxygenase 1
hr;	hours
HRP;	horseradish peroxidase
HSC;	haematopoietic stem cell
ICCS;	Intracellular cytokine staining
ICOS;	inducible costimulator
ICOSL;	ICOS ligand
iDC;	immature dendritic cell
IFN;	interferon
Ig;	immunoglobulin
I κ B	inhibitor of κ B
IKK;	I κ B kinase
IL;	interleukin
iNOS;	inducible nitric oxide synthase
IP ₃ ;	inositol 1,4,5-triphosphate
IPEX;	immunodeficiency, polyendocrinopathy, enteropathy, X-linked
syndrome	
IRAK;	Interleukin-1 receptor-associated kinase
IRF;	interferon regulatory factor
IS;	immunological synapse
ITAM;	immunoreceptor tyrosine-based activation motif
Itk;	IL-2- inducible T cell kinase
iTreg;	inducible T regulatory cell
IVR	intervening region
JNK;	c-Jun N-terminal kinase
kDa;	KiloDalton
Keap1;	Kelch-like ECH-associated protein 1
KO;	Knock out
L;	litre
LAT;	linker for activation of T cells
LC;	langerhans cell
Lck;	lymphocyte-specific protein tyrosine kinase
li;	invariant chain
LPS;	lipopolysaccharide
Lys;	lysine
m;	milli
M;	molar

mA;	milliamps
mAb;	monoclonal antibody
MAPK;	mitogen-activated protein kinase
MARCO;	macrophage receptor with collagenous structure
MEFs;	mouse embryonic fibroblasts
MCP-1;	monocyte chemotactic protein-1 also known as CCL2
mDC;	mature dendritic cell
MHC;	major histocompatibility complex
MIIC;	MHCII-rich compartment
min;	minutes
MIP-3 β ;	macrophage inflammatory protein-3 β
MLN;	mesenteric lymph node
MOPS;	3-(N-morpholino)propanesulfonic acid
mRNA;	messenger RNA
MS;	Multiple Sclerosis
MYD88;	Myeloid differentiation primary response gene (88)

NAC;	N-acetylcysteine
NADPH;	nicotinamide adenine dinucleotide phosphate
NaH ₂ PO ₄ ;	monosodium phosphate
NAPQI;	N-acetyl-p-benzoquinoneimine
Neh;	Nrf2-ECH homology
NER;	nuclear extraction reagent
NFAT;	nuclear factor of activated T cells
NF-E2;	nuclear factor erythroid 2
NF- κ B;	nuclear factor κ B
NK;	natural killer cell
NO;	nitric oxide
NOX;	NADPH oxidase
NP-40;	nonidet P-40
NQO1;	NAD(P)H:quinone oxidoreductase 1
Nrf2;	NF-E2 -related factor 2
nTreg;	natural occurring T regulatory cell

OH;	hydroxyl
\cdot OH;	hydroxyl radical
O ₂ ;	molecular oxygen
O ₂ $^{\cdot -}$;	superoxide anion radical
OVA;	ovalbumin

PAMPs;	pathogen-associated molecular patterns
PBMC;	peripheral blood mononuclear cell
PBS;	phosphate-buffered saline
PCR;	polymerase chain reaction
pDC;	plasmacytoid DC
PE;	phycoerythrin
PFA;	paraformaldehyde

PHA;	phytohemagglutinin
PI3K;	phosphatidyl inositol 3-kinase
PIP;	phosphatidylinositol 4-phosphate
PIP ₂ ;	phosphatidylinositol 4,5-biphosphate
PIP ₃ ;	phosphatidylinositol 3,4,5-triphosphate
PKC;	protein kinase C
PLC;	phospholipase C
PM;	particulate matter
PMA;	phorbol 12-myristate-13-acetate
PRR;	pathogen recognition receptor
PRX;	peroxiredoxin
PS;	phosphatidylserine
RA;	rheumatoid arthritis
RANTES;	Regulated on Activation, Normal T cell Expressed and Secreted
RBC;	red blood cell
RelA;	NF-κB p65 subunit
RIPA;	radio-immunoprecipitation assay
RO ₂ ·;	peroxyl radical
ROS;	reactive oxygen species
RORγt;	retinoic acid-related orphan receptor-γt or human RORC2
RPMI;	Roswell Park Memorial Institute-1640
RT;	room temperature
RUNX1;	Runt-related transcription factor 1
SCA-1;	stem cell antigen-1
SCZ;	sub-capsular zone
SD;	standard deviation
SDF-1;	stromal cell-derived factor-1
SEM;	standard error of the mean
SH2;	Src Homology 2
SLE;	Systemic lupus erythematosus
SLP-76;	SH2 domain-containing leukocyte phosphoprotein of 76 kDa
SOD;	superoxide dismutase
SP;	single positive
STAT;	Signal Transducer and Activator of Transcription
TAP;	transporter associated with antigen processing
T-bet;	T box expressed in T cells
tBHQ;	<i>tert</i> -butylhydroquinone
TBS;	Tris-buffered saline
TBST;	TBS-Tween
TCCD;	2, 3, 7, 8-tetrachlorodibenzo-p-dioxin
TCR;	T cell receptor
T _{CM} ;	central memory T cell
T _{EM} ;	effector memory T cell
Teff;	effector T cell

Tfh;	CD4 T follicular helper cell
TGFβ;	transforming growth factor β
Th;	CD4 T helper cell
TLR;	Toll-like receptor
TNFα;	tumour necrosis factor α
TNFR-1;	tumour necrosis factor receptor-1
TRAF-6;	TNF receptor-associated factor 6
Treg;	T regulatory cell
TRX;	Thioredoxin
TRXR;	Thioredoxin reductase
TSLP;	thymic stromal lymphopoietin
UV;	Ultraviolet
V-ATPase;	vacuolar-type H ⁺ -ATPase
VSIG ₄ ;	V-set and immunoglobulin domain containing 4
V region;	variable region
v/v;	volume/volume
WCL;	whole cell lysate
WHIM; syndrome	Warts, Hypogammaglobulinemia, Infections, and Myelokathexis
w/v;	weight/volume
XO;	xanthine oxidase
XRE;	xenobiotic response element
ZAP-70;	Zeta-chain-associated protein kinase 70

CHAPTER ONE

GENERAL INTRODUCTION

1.0 INTRODUCTION

1.1 The Immune System

Multi-cellular animals are continuously threatened by a diverse range of pathogenic and innocuous bacteria, fungi, viruses and parasites. The immune system is geared towards recognising the potential pathogens to which it is exposed to and thus defend the host against infection. Furthermore, this system must be able to discriminate between the animal's own cellular components (i.e. self) and that of an invading pathogen (i.e. non-self). Failure to do so gives rise to detrimental inappropriate immune responses which underpin the pathogenesis of autoimmune diseases such as diabetes and Multiple Sclerosis (MS).

The immune system is typically divided into two parts, the innate and the adaptive. Each division of the immune system is made up of both cellular and humoral components. Although the innate and adaptive arms functionally complement one another, they differ significantly in their capacity to recognise and subsequently eradicate infectious pathogens. The following introduction gives an overview of the innate and adaptive immune system with a focus on dendritic cells and T lymphocytes.

1.2 Innate immunity

The innate immune system is comprised of physical cellular barriers (e.g. skin and mucosal epithelia), humoral components (e.g. complement system and anti-microbial peptides), and innate immune cells that provide the initial, non-specific defence against invading pathogens. In general, innate immune cells including monocytes, macrophages, neutrophils, basophils, eosinophils, mast cells and conventional dendritic cells (cDCs) arise from common myeloid progenitors (CMPs) in the bone marrow. Other innate immune cells namely natural killer (NK) cells and particular subtypes of dendritic cells arise from bone marrow-derived common lymphoid progenitors (CLPs) (Beutler, 2004). The physiological and pathophysiological functions of the various types of innate immune cells are outlined in **Table 1.1**.

Cell type	Function and features	Pathophysiology	Reference
Basophil	<ul style="list-style-type: none"> • Morphologically similar to mast cells • Activation via cross-linkage of Fc_εRI with IgE resulting in degranulation • Provide anti-parasitic immunity: <ul style="list-style-type: none"> - Release granules containing histamine and proteases - Produce IL-4, IL-13 	Helminth infection, asthma, allergic rhinitis, atopic dermatitis	(Stone et al., 2010)
Dendritic cell (DC)	<ul style="list-style-type: none"> • Most Potent APC • Initiate and modulate adaptive immune responses • Involved in anti-viral, anti-parasitic, anti-bacterial, anti-fungal and anti-tumour immunity • Maintenance of T cell tolerance 	SLE, systemic sclerosis, RA, asthma, psoriasis, atopic dermatitis, hypersensitivity reactions, measles, cancer, type 1 diabetes	(Ueno et al., 2007; York, 2011; Dorner, 2012)
Eosinophil	<ul style="list-style-type: none"> • Activated by cross-linkage of FcγRII with IgG resulting in degranulation • Provide anti-parasitic immunity: <ul style="list-style-type: none"> - Release cytolytic granule proteins - Produce IL-4, IL-5, IL-10, TNFα 	Helminth infection, allergic rhinitis, asthma, hyper-IgE syndrome, atopic dermatitis	(Stone et al., 2010)
Macrophage	<ul style="list-style-type: none"> • Activated by PAMPs via TLR and opsonised bacteria via FcR • Professional phagocyte and APC capacity • Involved in cellular homeostasis via ingestion of apoptotic cells • Involved in anti-bacterial immunity: <ul style="list-style-type: none"> - Engulfment of microbes and lysis via NOX-respiratory burst mechanisms. - Produce IL-1β, TNFα, IL-12, IL-6, IL-8 	Gaucher disease, Atherosclerosis, systemic sclerosis, SLE, sepsis	(Morel et al., 1991; Naito, 2008; York, 2011; Dorner, 2012) (Tzima et al., 2009)

Macrophage (continued)	<ul style="list-style-type: none"> Involved in anti-viral immunity: <ul style="list-style-type: none"> Secrete IFNβ preventing intracellular viral replication 		
Mast cell	<ul style="list-style-type: none"> Activated by cross-linage FcϵRI with IgE resulting in degranulation Involved in anti-parasitic immunity <ul style="list-style-type: none"> Release granules containing histamine and proteases Produce TNFα, IL-5, IL-3 	Helminth infection, anaphylaxis, allergic rhinitis, asthma, mastocytosis, systemic sclerosis, atopic dermatitis	(Stone et al., 2010; York, 2011)
Monocyte	<ul style="list-style-type: none"> Precursor reserve pool for macrophages and DCs (under inflammatory conditions) Professional phagocyte but low APC capacity as <i>bona fide</i> monocyte Involved in cellular homeostasis via ingestion of apoptotic cells Involved in anti-bacterial immunity: <ul style="list-style-type: none"> engulfment of microbes and killing via iNOS activity and ROS production Produce TNFα, IL-1β, IL-6, IL-10 	RA, Atherosclerosis, systemic sclerosis, Crohn's disease, HIV, sepsis, asthma	(Auffray et al., 2009; York, 2011)
Natural Killer cell (NK)	<ul style="list-style-type: none"> Provide anti-tumour and anti-viral immunity: Target and lyse abnormal/viral infected cells which have low/absent expression of self-MHCI cell surface molecules Provide anti-bacterial immunity: Recognise and lyse antibody coated targets via FCR activation. Lysis via release of cytolytic granules containing perforin and granzyme B 	systemic sclerosis, MS, RA, type 1 diabetes, cancer, leukaemia	(Liang and Graham, 2008; Wu and Van Kaer, 2009; York, 2011)

Neutrophil	<ul style="list-style-type: none"> • Activated by PAMPs via TLR via and opsonised bacteria via FcR • Professional phagocyte and APC capacity • Involved in cellular homeostasis via ingestion of apoptotic cells • Involved in anti-bacterial immunity: <ul style="list-style-type: none"> - Engulfment of microbes and lysis via NOX respiratory burst mechanisms. - Produce IL-1β, TNFα, IL-12, IL-6, IL-8 	CGD disease, myelokathexis syndrome (WHIM), SLE, sepsis	(Dale et al., 2008; Dorner, 2012)
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Table 1.1 Innate immune cells and their roles in health and disease.

APC: antigen presenting cell; CGD: Chronic granulomatous disease; FcR: Fc receptor which binds to Fc portion present in tail region of secreted immunoglobulin; HIV: Human immunodeficiency virus; IFN β : interferon- β ; Ig: Immunoglobulin or antibody secreted by activated B-cells IL-: interleukin; iNOS: inducible nitric oxide synthase; MHC: major histocompatibility complex; MS: Multiple sclerosis; NOX: nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; PAMPs: pathogen-associated molecular patterns; RA: Rheumatoid arthritis; ROS: reactive oxygen species; SLE: Systemic lupus erythematosus; TLR: Toll-like receptor; TNF α : Tumor necrosis factor- α

Innate immune cells migrate via the blood stream and distribute throughout the body which helps in the rapid identification and efficient removal of potential infectious pathogens. These cells are activated upon recognition of conserved molecular components, known as pathogen-associated molecular patterns (PAMPs) (e.g. lipopolysaccharide (LPS) found in the bacterial cell wall of gram negative bacteria), via the expression of pathogen recognition receptors (PRRs) including Toll-like receptors (TLRs) (Beutler, 2004). These receptors are germline-encoded and recognise a limited number of molecules that are shared by numerous infectious agents. Engagement of PRRs result in a variety of immediate effector responses that endeavour to directly disable the harmful pathogen usually within the first 12 hours of infection (Murphy et al., 2012). A prime example is the activation of dectin-1, a member of the C-type lectin receptor (CLR) superfamily,

that is present on phagocytic myeloid cells including neutrophils and macrophages (Geijtenbeek and Gringhuis, 2009). Dectin-1 binds to β -glucans present in fungal cell walls, certain bacteria and plants, resulting in the phagocytosis and proteolytic destruction of the pathogen (Lee and Kim, 2007). Activation of dectin-1 also triggers the release of proinflammatory cytokines e.g. IL-1 β and chemokines e.g. CXCL8, which not only promote inflammation, but also instigate the recruitment of neutrophils and macrophages at the site of infection. This results in the amplification of the inflammatory response and effective removal of the pathogen (Murphy et al., 2012). Neutrophils, macrophages and in particular dendritic cells (DCs) are termed antigen presenting cells (APCs), which possess a unique ability to sense and engulf pathogens from sites of infection that are then processed and presented to adaptive immune cells in secondary lymphoid organs (**Table 1.1**). Proinflammatory cytokine release from innate immune cells, play a crucial role in priming APCs for efficient antigen presentation, APC-mediated adaptive immune cell activation and subsequent differentiation. This highlights the pivotal role of the innate immune system in complementing and modulating adaptive immune responses (Maldonado-Lopez et al., 1999; Tse et al., 2007; Abi Abdallah et al., 2011).

The innate immune system is not without its limitations. The PPRs are restricted to recognising only common microbial structural features and thus are limited in perceiving a narrow range of potential pathogenic insults. Furthermore, these receptors cannot provide antigen-specific protection to invading pathogens and thus fail to prevent re-infection (Beutler, 2004). In contrast to the innate arm of the immune system, the adaptive immune system specifically recognises and provides protective immunity against a diverse array of invading pathogens, but more importantly, it provides long lasting protection against re-infection due to its unique feature, immunological memory. Although adaptive immune responses are long lasting, they are delayed in their initiation. Therefore, the adaptive arm must work in concert with the rapid responses of the innate immune system to ensure both the immediate and long term protection against pathogenic invasion.

1.3 Adaptive Immunity

The adaptive immune system is organized around B and T lymphocytes, which have the ability to recognise a myriad of invading pathogens and can mount a specific immune response. The great diversity in antigen specificity among lymphocytes is determined by the structure of the B and T cell receptors' (BCR and TCR) antigen-binding site, whose structural segments are a result of somatic recombination of antigen receptor genes, together with palindromic and random nucleotide addition during development. Naïve T cells and B cells reside in secondary lymphoid tissues such as the spleen, lymph nodes and Peyer's patches, where they await contact with circulating DCs. The DCs migrate to lymphoid tissues and present captured antigens from the periphery to these adaptive immune cells. The recognition of antigens by the TCR/BCR leads to T/B cell clonal expansion and affinity maturation. Clonal expansion gives rise to a large pool of antigen-specific T or B cells, some of which remain dormant in the body after the infection has been resolved. This gives rise to immunological memory which permits a rapid response upon second exposure to the same antigen (Parkin and Cohen, 2001).

B cells are critical mediators of humoral immunity owing to their distinctive capacity to produce antigen-specific antibodies (also known as immunoglobulins (Ig)). During B cell development in the bone marrow, a competent rearranged BCR is formed which is composed of an antigen-specific transmembrane Ig (Pieper et al., 2013). B cells migrate to follicular regions of peripheral lymphoid organs where they await recognition of their respective antigen. Generally, B cells require T cell help for competent B cell activation and differentiation. Once the B cell has become fully activated, it undergoes processes of somatic hypermutation and affinity maturation, resulting in the clonal expansion of high affinity antigen-specific, antibody-secreting plasma cells and memory cells (LeBien and Tedder, 2008). In parallel, cytokines secreted from the T helper cells induce Ig isotype switching from IgM, IgD to secondary isotypes IgA, IgE and IgG (Parkin and Cohen, 2001). Secondary isotype secretion results in a variety of humoral-mediated adaptive immune responses including the enhancement of innate immune cell responses and ultimate clearance of infection. However, inappropriate production of these

antibodies can underlie pathological processes, as is the case for IgE and allergic disease (**Table 1.1**).

T cells play vital roles in mediating both cellular and humoral-specific adaptive immune responses. There are various types of T cells present within the body as discussed later in this chapter, each with distinctive functions that provide protective anti-viral, anti-tumour, anti-parasitic, anti-bacterial and anti-fungal immunity. However, T cell dysregulation is an underlying cause of a variety of autoimmune and allergic disorders. In order to fully understand the function of the T cell, it is imperative to gain an insight into their development and importantly their activation by the potent innate APC, the DC.

1.4 T cell development

T Lymphocytes possess a diverse TCR repertoire enabling recognition of a diverse range of antigens with high specificity. How they achieve this crucial function for targeting a variety of pathogens, lies within the events associated with their development and differentiation into mature T cells in the thymus as illustrated in **Figure 1.1**. Initially, T lymphocytes arise from Common lymphoid progenitors (CLPs) in the bone marrow which migrate to the thymus via the blood stream. Within the thymus, CLPs receive direct instructions to differentiate into early committed T cells termed double negative (DN) which lack expression of co-receptors CD4 and CD8 (CD4⁻CD8⁻). As DN cells travel through the thymic cortex and the cortical sub-capsular zone (SCZ), they undergo four stages of differentiation as distinguished by their expression of CD44 and CD25; CD44⁺CD25⁻ (DN1), CD44⁺CD25⁺ (DN2), CD44⁻CD25⁺ (DN3) and CD44⁻CD25⁻ (DN4) (**Figure 1.1**)(Carpenter and Bosselut, 2010). Interactions with thymic stromal cells facilitate the progression of the immature thymocytes through stages DN1- DN3 (Maillard et al., 2005). At the DN3 stage, T cells undergo T cell receptor β (TCR β) chain somatic gene rearrangement also known as β -selection. The recombined TCR β chain couples with an invariant pre-TCR α chain resulting in the assembly of the pre-TCR. Competent signal transduction through the pre-TCR facilitates progression from DN3-DN4 (Carpenter and Bosselut, 2010). Robust cell proliferation occurs during DN4 maturation,

followed by TCR α chain somatic rearrangement, resulting in the formation of a functional heterodimeric TCR $\alpha\beta$. These cells begin to co-express CD4 and CD8, ultimately giving rise to the CD4⁺CD8⁺ double positive (DP) thymocyte population (**Figure 1.1**)(Germain, 2002). The survival of these DP cells and subsequent maturation into single positive CD8 and CD4 T cells, is dependent upon their interaction with self-peptides displayed by residual cortical epithelial cells in the context of MHCI and MHCII molecules, respectively (Germain, 2002). Double positive T cells containing TCRs that interact poorly with self-peptide:MHC complexes, do not receive survival signals and are thus deleted from the T cell repertoire via programmed cell death mechanisms. Double positive T cells that do recognise self-peptides:MHC complexes presented by epithelial cells are positively selected (Germain, 2002). As a consequence of positive selection, DP T cells lose co-expression of CD4 or CD8 and subsequently migrate to the thymic medulla where they reside as single positive (SP) CD8 and CD4 T cells, respectively (**Figure 1.1**)(Germain, 2002). Following positive selection, SP T cells which recognise self-peptide:MHC complexes presented by medullary epithelial cells or thymic DCs too avidly and thus have the potential to initiate peripheral autoimmune responses, receive strong cell death signals resulting in their deletion. This process is known as negative selection which underlies T cell central tolerance (Germain, 2002). Mature non-autoreactive SP CD4 and CD8 T cells are then free to migrate to peripheral secondary lymphoid organs such as the spleen and lymph nodes, where they await activation from APCs. Natural occurring immunosuppressive CD4⁺CD25⁺Foxp3⁺ T regulatory cells (nTregs) also develop within the thymus but the precise signals involved in their development remain elusive (Sakaguchi, 2004). They have been shown to be derived from SP CD4 T cells in the thymic medulla (**Figure 1.1**) (Sakaguchi, 2004). T regulatory cells play an imperative role in maintaining T cell homeostasis and in the prevention of autoimmune responses as discussed in **section 1.11.4**.

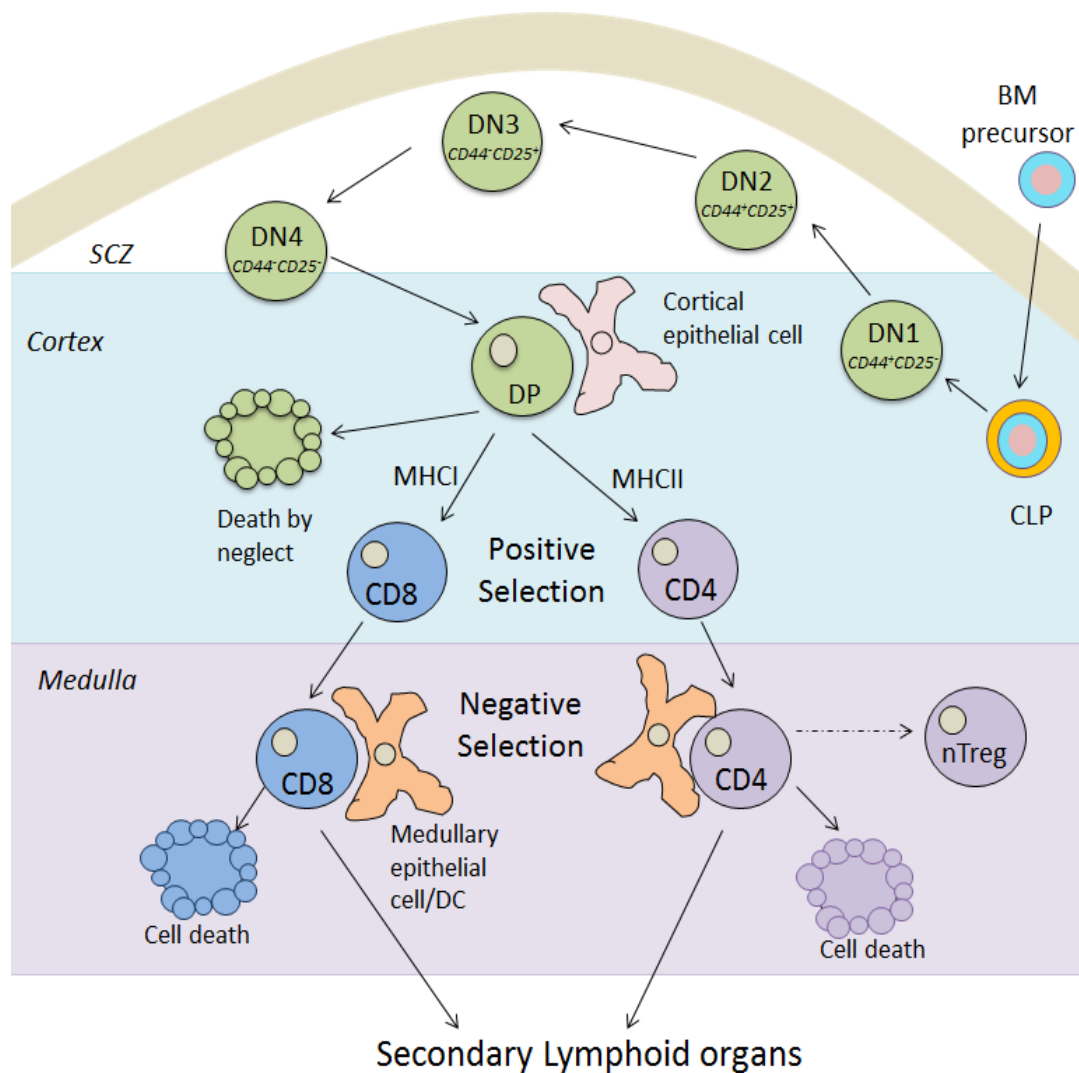


Figure 1.1 Schematic diagram of T cell development within the thymus

Immature thymocytes undergo consecutive phases of differentiation and endure stringent positive and negative selection processes as they travel through the thymus. This results in the selection of mature non-self reactive CD4 and CD8 T cells which subsequently egress from the thymus and migrate to peripheral lymphoid organs. BM: bone marrow; CLPs: common lymphoid progenitors; DN: double negative; DP: double positive; MHC: major histocompatibility complex; SCZ: sub-capsular zone; SP: single positive; TCR: T cell receptor.

1.5 Dendritic cells

Dendritic cells are specialised APCs present in the blood, skin, peripheral organs e.g. gut, lung and liver, and lymphoid organs e.g. the spleen and thymus. They are responsible for the initiation and modulation of primary adaptive immune responses (Banchereau et al., 2000). They exist in two functional states; immature steady state DCs (iDCs) and mature DCs (mDCs) as determined by their morphology, phenotypic expression of cell surface proteins which promote T cell activation (known as co-stimulatory molecules), chemokine expression and their immune function as shown in **Table 1.2** (Banchereau et al., 2000).

Feature	Immature DC (iDC)	Mature DC (mDC)
Morphology	Spherical shape	Large cytoplasmic protrusions (dendrites)
Residence	Periphery	Secondary lymphoid organs
Function	<ul style="list-style-type: none">- Antigen capture- High phagocytosis- Poorly immunogenic- Maintenance of CD8 T cell tolerance	<ul style="list-style-type: none">- Antigen presentation- Low phagocytosis- Highly immunogenic- Modulation of adaptive immune responses
Chemokine expression	<ul style="list-style-type: none">- High CCR1, CCR5, SDF-1, IL-8- Low CCR7, MIP-3β	<ul style="list-style-type: none">- Low CCR1, CCR5, SDF-1, IL-8- High CCR7, MIP-3β
Co-stimulatory receptor expression	<ul style="list-style-type: none">- Low CD80, CD86, CD40, CD83- High intracellular MHCII	<ul style="list-style-type: none">- High CD80, CD86, CD40, CD83- High cell surface MHCII

Table 1.2. Contrasting roles and features of immature and mature DCs.

CCR: chemokine (C-C motif) receptor; CD: cell surface molecule; IL-8: interleukin-8; MHCII: major histocompatibility complex II; MIP-3 β : Macrophage inflammatory protein-3 β also known as chemokine (C-C motif) ligand 19 (CCL19); SDF-1: stromal cell-derived factor-1
Adapted from (Banchereau et al., 2000)

Dendritic cells arise from haematopoietic stem cells (HSCs) in the bone marrow. The development pathways for DCs are complex as they are derived from both CLP and

CMP lineages (Kushwah and Hu, 2011). The resultant heterogenic DC population is classified into a variety of subtypes in the mouse and human, each with their own specific set of cell surface molecules, anatomical location and functional response in the body (Villadangos and Schnorrer, 2007). In spite of their diversity, they collectively share a common function in the processing and presentation of antigens to the adaptive immune system, thus providing a bridge between the innate and adaptive immune system. Dendritic cells can be broadly classified into two major subtypes; plasmacytoid DCs (pDCs) and conventional DCs (cDCs) (Barchet et al., 2005; Villadangos and Schnorrer, 2007). Plasmacytoid DCs are distinctive from the cDC subtype due to their low phagocytic capacity, unique cytokine profile and low expression of cell surface CD11c and MHCII. Plasmacytoid DCs are present throughout the periphery, blood and lymphoid organs and specialise in the recognition and presentation of viral-derived antigens. Moreover, they secrete large amounts of type I interferons (IFNs), IFN α and IFN β , upon activation, thus play an imperative role in instructing adaptive anti-viral immune responses (as reviewed in (Reizis et al., 2011)). In contrast, cDCs encompasses all other types of DCs present in lymphoid and non-lymphoid tissues that express high levels of CD11c and MHCII. In contrast to pDCs, cDCs are more efficient at capturing, processing and presenting antigens to naive T cells and directing the nature of the adaptive immune response elicited (Merad et al., 2013). Murine cDCs can be further subdivided into lymphoid organ-resident DCs found in the spleen, thymus and lymph nodes, migratory DCs subsets e.g. langerhans cells present in the skin and interstitial DCs which are in peripheral organs including the gut (Kushwah and Hu, 2011). The precise phenotype for each cDC subset is reviewed elsewhere (Merad et al., 2013). Migratory DCs are named such due to unique ability at acquiring antigens from the periphery and migrating via the blood to lymphoid organs (Villadangos and Schnorrer, 2007). The bone-marrow derived DCs utilised in this thesis are akin to this non-lymphoid migratory CD11c⁺MHCII⁺ subset. It is important to note that the function of the cDCs differ depending on the location. For example, splenic-resident CD8⁺ DCs are highly efficient at cross presenting cell-associated antigens to CD8 T cells and initiating CD8 T cell responses whereas DCs present in the gut play an important

role in inducing tolerance and directing CD4 T regulatory cell polarisation (Maldonado-Lopez et al., 1999; Belz et al., 2005; Scott et al., 2011).

1.6 Dendritic cells: drivers of the immune response

Dendritic cells survey the peripheral environment for potential infectious pathogens subsequently directing cells of the adaptive immune system to execute finely tuned immune responses, ultimately resulting in the clearance of infection and are thus important sentinels of the immune system (Banchereau et al., 2000). In the periphery, DCs reside in an antigen-capturing immature state sampling their environment for potential pathogenic invasion. The iDC can ingest exogenous pathogens, dying and infected cells through engagement of their endocytic and phagocytic receptors. Receptors engaged include dectin-1 for β -glucans, Fc γ R for IgG opsonised bacteria) and phosphatidylserine receptor (PS), scavenger receptor CD36, and C-type lectin domain family 9A (CLEC9A, also known as DNGR-1) for apoptotic and necrotic cells (Dalgaard et al., 2005; Bratton and Henson, 2008; Geijtenbeek and Gringhuis, 2009; Sancho et al., 2009; den Dunnen et al., 2012). Receptor engagement consequently induces a variety of actin-dependent phagocytic processes facilitating the efficient internalisation of the pathogen and formation of the phagosome (Underhill and Goodridge, 2012).

In the absence of infection, host-associated (self) antigens are constitutively acquired, processed and presented by iDCs to T cells in lymphoid organs (Wilson et al., 2004). Presentation of self-antigens is critical for maintaining T cell tolerance. This instructs the deletion of those autoreactive T cells that have escaped normal central tolerance mechanisms within the thymus, thus dampening detrimental autoimmune responses and the occurrence of autoimmune diseases (**Table 1.2**) (Kurts et al., 1997; Kurts et al., 1998). How the DC signals the presence of antigens associated with pathogenic infection i.e. non-self-antigens is driven through additional cues provided by the selective engagement of TLRs (Mellman and Steinman, 2001; Schulz et al., 2005; Blander and Medzhitov, 2006b; den Dunnen et al., 2012). Upon encounter with an infectious pathogen, molecular patterns associated with the invading pathogen (PAMPs) are recognised by the TLRs. The TLR

family is comprised of 10 members in humans and 12 in mice and can collectively recognise common constituents derived from multifarious pathogens including bacteria, parasites, viruses and fungi (Kawai and Akira, 2011). Toll-like receptor signalling results in the activation of the mitogen-activated protein kinases (including p38 MAPK, JNK and ERK1/2) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signalling pathways which promote the nuclear translocation of transcription factors, activator protein 1 (AP-1) and NF- κ B, respectively (Kawai and Akira, 2011). As a consequence, these transcription factors drive the transcription of a variety of genes involved in DC maturation including lymphoid-homing chemokine receptor CCR7, inflammatory cytokines e.g. IL-1 β , TNF α , IL-12 and IL-6, cell surface expression of MHCII and the upregulation of co-stimulatory receptors e.g. CD80, CD86 and CD40 (**Table 1.2**) (Sallusto et al., 1998; Ardeschna et al., 2000; Nakahara et al., 2006; Neves et al., 2009). The phenotypic and morphological changes associated with DC maturation alter the immune function of the dendritic cell from highly phagocytic iDCs efficient at antigen capture, to low phagocytic, highly immunogenic mDCs proficient at antigen presentation (**Table 1.2**). Consequently, mDCs efficiently migrate to lymphoid organs where they present processed antigens to naive T cells in such a context, resulting in the initiation of an adaptive immune response. Importantly, these events enable DCs to greatly influence the nature of the T cell response elicited by guiding naive T cell differentiation into functionally distinct effector subtypes as reviewed later in this chapter.

1.7 Dendritic cell antigen presentation

In order for a DC to present antigens to naive T cells, they must first process and load the antigen onto specialised proteins known as major histocompatibility complex (MHC) molecules (Trombetta and Mellman, 2005). These molecules are synthesised in the ER and are crucial for permitting the efficient presentation of the antigen at the surface of the DC for subsequent recognition by the TCR (Trombetta and Mellman, 2005). How the antigen is processed and presented depends upon the type of pathogen initially encountered. This also influences the type of T cell (CD4 or CD8) that becomes activated. In general, endogenous proteins generated

from within the cell or from intracellular viruses, parasites or bacteria derived from e.g. intracellular bacteria, viruses, self-defective ribosomal products (DRiPs) or misfolded proteins are processed and loaded onto MHC class I molecules, which are specifically recognised by CD8 T cells (Trombetta and Mellman, 2005). In contrast, internalised exogenous antigens derived from e.g. extracellular bacteria and parasites, are loaded onto MHC II molecules and presented to CD4 T cells (Trombetta and Mellman, 2005). However, there are exceptions to this rule as observed with exogenous dying or viral infected cell MHCI-mediated presentation to CD8 T cells, a unique process known as cross-presentation (**section 1.7.3**) (Trombetta and Mellman, 2005). The mechanistic basis of the various types of DC antigen processing and presentation are discussed below.

1.7.1 Endogenous classical MHCI pathway

Dendritic cells use the classical MHCI pathway to present processed cytosolic proteins (generated from within the cell or from intracellular viruses, parasites or bacteria) in conjunction with MHCI to naive CD8 T cells. This type of presentation relies on the cytosolic proteasomal machinery for efficient antigen processing, and transport of peptides from cytosol to the ER for efficient loading onto MHCI via the transporter associated with antigen processing (TAP) as illustrated in **Figure 1.2A**. Dendritic cells constitutively present antigens via this pathway independently of the maturation status of the DC. The distinction between DC presentation of self or non-self-antigenic peptides and the subsequent CD8 T cell response (tolerance vs. immunity) is dependent on the presence of DC maturation signals. Under normal physiological conditions, the absence of TLR ligands does not initiate DC maturation and keeps CD8 T cell in a tolerant state (Blum et al., 2013). However under viral infectious conditions, the virus enters the DC and produces dsRNA as it replicates. dsRNA activates intracellular TLR3 contained within endosomal compartments, resulting in DC maturation (Kawai and Akira, 2011). The MHCI:peptide complexes that are transported to the surface of the DC in this context will result in an antigen-specific CD8 T cell immune response.

1.7.2 Exogenous MHCII pathway

Dendritic cells present exogenous peptides derived from internalised extracellular bacteria, fungi and parasites through the exogenous MHCII pathway. Processed antigenic peptides are then presented in the context of MHCII molecules to CD4 T cells. This type of presentation relies on the phagosomal vesicular pathway for the processing and transportation of exogenous antigenic peptides onto translocated MHCII molecules. Unlike endogenous MHCI presentation, efficient exogenous MHCII presentation only proceeds in mature DCs following TLR engagement (Blum et al., 2013). With reference to extracellular *Escherichia coli* bacterial infection, LPS is recognised by TLR4 which promotes the phagocytosis and internalisation of the bacteria into the phagosome. The phagosome then enters a sequential vesicular pathway in which its contents are subsequently delivered to increasingly proteolytic enzyme-rich late endosomal and lysosomal compartments, a process known as phagosome maturation (Blander and Medzhitov, 2006a). Transportation of the MHCII molecule itself relies on the activity of the protease cathepsin S which is also activated upon TLR signalling. The precise details of MHCII presentation are illustrated in **Figure 1.2B**. (Blander and Medzhitov, 2006b).

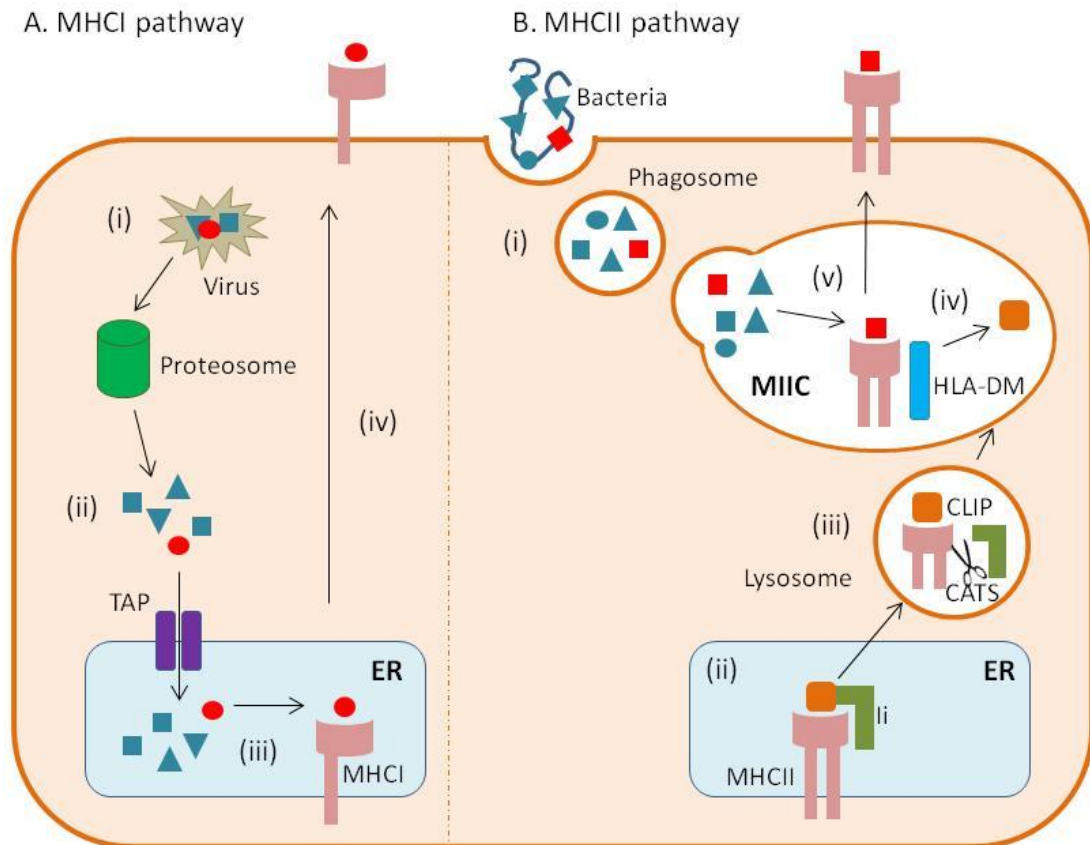


Figure 1.2. DC antigen presentation pathways

A. Endogenous classical MHC I pathway (i) Intracellular viral proteins are ubiquitinated and degraded by the proteasome. (ii) Peptides are transported from the cytosol to the ER via TAP. (iii) Antigenic peptides are loaded onto MHC I molecules with the ER. (iv) The MHC I:peptide complex is then actively transported from the ER to the cytosol and ultimately displayed on the surface of the DC to naive CD8 T cells. **B. Exogenous MHC II pathway** (i) Bacterial pathogens are internalised resulting in the formation of the phagosome. This fuses with the late endosomal compartment, MIIC where the bacteria are proteolytically degraded into smaller antigenic peptides. (ii) Within the ER, MHC II molecules are formed which are stabilised by the binding of type II membrane spanning chaperone protein, li. (iii) MHC II:li complex is transported to lysosomes where li is cleaved to a smaller peptide CLIP by cathepsin S. (iv) The lysosome fuses with the MIIC containing the chaperone protein, HLA-DM which dissociates CLIP from MHC II. (v) Specific antigenic peptide contained within the MIIC are loaded onto the MHC II and transported to the surface of the DC where it is displayed to naive CD4 T cells. CATS: cathepsin S; CLIP: MHC class II–associated invariant-chain peptide, DC: dendritic cell; ER: endoplasmic reticulum; HLA-DM: human leukocyte antigen-DM; li: invariant chain; MHC: major histocompatibility complex; MIIC: MHC class II-rich compartment; TAP: transporter associated with antigen processing.

1.7.3 Cross-presentation

Certain DC subsets notably the CD8⁺ lymphoid DCs can uniquely present exogenous cell-associated antigens from dying or infected cells in the context of MHCI molecules to naive CD8 T cells, a phenomenon known as “cross-presentation” (den Haan et al., 2000; Belz et al., 2005). In the case of intracellular viral infection, presentation of MHCI:peptide complex to CD8 T cells relies on the DC to be infected itself as illustrated with the classical endogenous MHCI pathway. However, viruses can utilise mechanisms to severely impede MHCI presentation in infected DCs thus evading cytotoxic CD8 T cell immunity (Nopora et al., 2012). Cross priming mechanisms provides a means by which uninfected DCs can initiate CD8 T cell immune responses through the processing of cell-associated antigens derived from internalised viral infected cells (Nopora et al., 2012). Furthermore, it is essential for priming CD8 T cell anti-tumour responses to exogenous tumour self-antigens expressed by dying cells (Hoffmann et al., 2000). Unlike, the exogenous MHCII pathway, cross-presentation occurs in nascent phagosomes (Houde et al., 2003) or early endosomes typified by their neutral pH and generally relies on transport of their exogenous material into the cytosol for proteasomal degradation (Blum et al., 2013). Therefore, phagosomal maturation into late proteolytically active endosomal compartments is not required. Similarly to MHCI presentation, cross-presentation is a constitutive process in iDCs and plays an imperative role in maintaining CD8 T cell tolerance (Kurts et al., 1998). This proceeds through iDC presentation of extracellular self-antigens in the context of MHCI to autoreactive CD8 T cells, which results in their deletion from the T cell repertoire and ultimately prevents the initiation of autoimmune responses (Kurts et al., 1997; Kurts et al., 1998; Heath and Carbone, 2001). The differential pathways involved in cross-presentation are illustrated in **Figure 1.3**. Take for example the cross-presentation of cell-associated antigens derived from apoptotic or necrotic cells. These cells are internalised through phagocytic mechanisms into phagosomes and are partially degraded, after which they are expelled from the phagosome to the cytosol, ubiquitinated and directed for proteasomal degradation (**Figure 1.3A**, black arrows) (Blum et al., 2013). The resultant antigenic peptides are subsequently transported to the ER via

TAP where they are loaded onto MHCI molecules and transported to the surface of the cell (Kovacsovics-Bankowski and Rock, 1995). The phagosome itself can also acquire the optimum components for efficient exogenous MHCI peptide loading via fusion with ER-derived vesicles (**Figure 1.3B**, green arrows). In this respect, the acquired dying cell is partially degraded in the ER-fused phagosome and the resultant proteins are transported from the fused phagosome to the cytosol via the transporter Sec61, where they are directed to the proteasome for further degradation. The resultant peptides are then re-translocated back into the phagosome via TAP where the peptide is loaded onto the MHCI and transported to the surface of the DC (Houde et al., 2003). Cross presentation can also proceed via a TAP and proteasome-independent mechanism (**Figure 1.3C**, red arrows) (Bachmann et al., 1995). In this process, acquired dying cells are transported via the phagosome to vacuolar post-golgi compartments where they are broken down into smaller peptide fragments via proteases. Recycled MHCI from the surface of the DC are acquired within these phagocytic vesicles. The peptide is loaded onto the MHCI and the MHCI:peptide complex is then transported to the surface of the DC (Trombetta and Mellman, 2005).

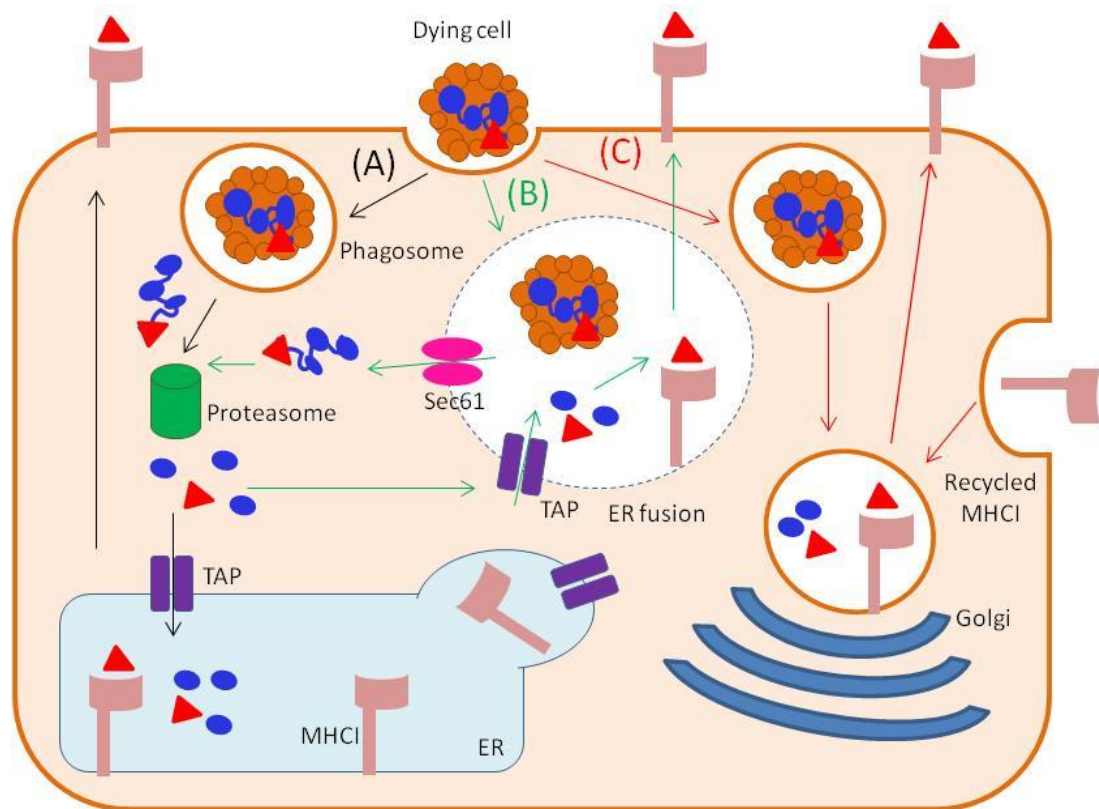


Figure 1.3 DC cross presentation pathways

Cross presentation of exogenous dying cell-associated antigens in conjunction with MHC molecules to naïve CD8 T cells can proceed via three mechanisms **(A)** proteasome- and TAP dependent pathway **(B)** proteasome- and TAP-dependent ER fusion **(C)** proteasome and TAP independent processing via the vacuolar post-golgi compartment. ER: endoplasmic reticulum; MHC: major histocompatibility complex; TAP: transporter associated with antigen processing.

1.8 DC-mediated antigen-specific T cell activation

Once the antigen is processed and displayed on the DC cell surface, it is now available for specific recognition by naïve T cells in lymphoid organs. In order for competent T cell activation to occur, three activation signals are required (**Figure 1.4**). The T cell must specifically recognise the peptide:MHC presented by the mDC via its distinct TCR (signal 1) (Murphy et al., 2012). CD8 and CD4 TCRs will specifically recognise peptides complexed to MHCI and MHCII molecules, respectively. Co-stimulatory receptor engagement between CD28 on T cells and CD80/86 on DCs comprises the second critical signal (signal 2) necessary for T cell

activation (Murphy et al., 2012). Co-stimulatory receptor signal transduction lowers the threshold for T cell activation, prevents T cell anergy, enhances T cell survival via up regulation of anti-apoptotic protein Bcl-x_L and facilitates IL-2 cytokine secretion (Bachmann et al., 1997; Okkenhaug et al., 2001; Acuto and Michel, 2003). It is important to note that as iDCs express low levels of co-stimulatory receptors, they present the antigen to T cells in the absence of signal 2, thus resulting in T cell tolerance (Kurts et al., 1998). Signal 3 represents cytokines secreted by the DC itself e.g. IL-12 or from other innate immune cells at the site of infection. This tertiary signal is vital for the functional differentiation of CD4 and CD8 T cells into mature effector T cells. Once all signals are in place, this will result in the induction of competent antigen-specific naïve T cell activation. T cell activation proceeds via the triggering of complex tyrosine kinase cascades and other signalling pathways including p38 MAPK as reviewed in (Smith-Garvin et al., 2009). This results in the downstream activation and nuclear translocation of transcription factors Nuclear factor of activated T cells (NFAT), NF-κB and AP-1, which work in concert in the transcriptional upregulation of an array of genes responsible for T cell survival, proliferation, differentiation and cytokine secretion such as IL-2 (Macian et al., 2001; Hayden and Ghosh, 2011).

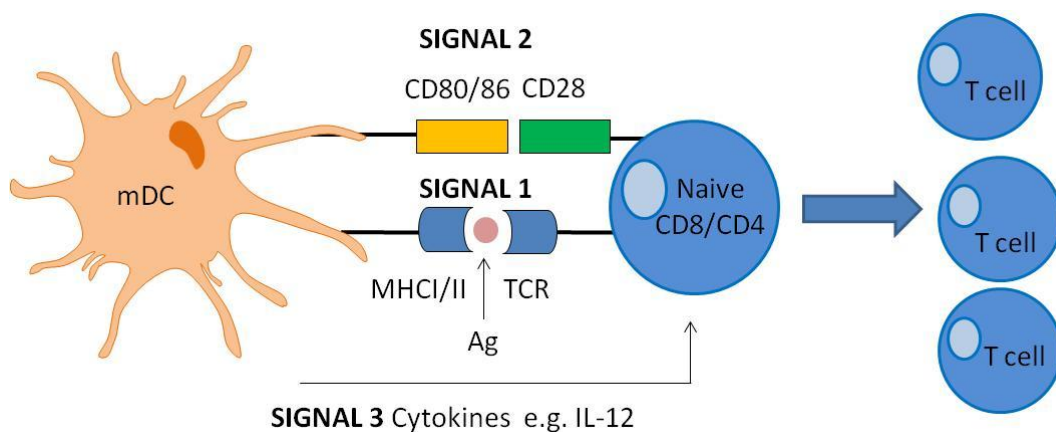


Figure 1.4. Schematic diagram of DC-mediated T cell activation within peripheral lymphoid organs

Ag: antigen; IL-12: interleukin-12; mDC: mature DC; MHC: major histocompatibility complex; TCR: T cell receptor.

1.9 T cells and the immune response

Once a naïve T cell has recognised a specific antigen presented by the DC and is consequentially fully activated, it will undergo several rounds of proliferation. This results in its clonal expansion and functional differentiation into effector T cells. Naïve T cells are identified by their distinct cell surface expression of CD62L and chemokine receptors CCR7 and CXCR4 (Bromley et al., 2008; Kesarwani et al., 2012). Expression of CD62L, CXCR4 and CCR7 allow entry and residence in lymphoid tissues. Differentiation into effector cells is associated with down regulation of the aforementioned surface markers thus enabling them to leave the lymph node (Kesarwani et al., 2012). Migration to peripheral tissues and sites of infection through the blood stream and lymphatics is achieved through the upregulation of chemokine receptors such as CXCR3, CCR4, CCR5 and CCR6 (Bromley et al., 2008). Herein they perform specific adaptive immune responses upon re-exposure to the antigen and ultimately result in the clearance of infection and attainment of immunological memory. There are two major types of effector T cells, cytotoxic CD8 T cells (CTLs) and CD4 T helper cells. The type of T cell that is activated by an APC, depends on whether the pathogen was processed via the endogenous classical MHC I pathway or the exogenous MHC II pathway. CD8 CTLs and CD4 T cells are fundamental for providing defence against invading intracellular and extracellular pathogens, respectively. As stated previously, functional differentiation into competent effector T cells is highly dependent upon the cytokines present within their microenvironment (**Figure 1.4**, signal 3). For CD8 T cells, IL-12 acts as the third signal since its absence limits the capacity of the CD8 T cells to proliferate and to produce IFN γ (Curtsinger et al., 1999). Abrogation of this third signal also results in CD4 hyporesponsiveness (Tse et al., 2007). At the peak of the immune response, a minor population of cells diverge into memory T cells as typified by their expression of IL-7R (Zielinski et al., 2011). In contrast to naïve and effector cells, memory cells rely solely on cytokines IL-7 and IL-15 for their survival (Surh and Sprent, 2008). Therefore, once the infection and its associated antigen have cleared, the majority of T cells die en masse via various pro-apoptotic mechanisms within 7 days post-infection. The small population of memory cells which survive possess the

immunological memory of that specific antigen (Zimmerer et al., 2012). Unlike short-lived effector cells, antigen-experienced memory cells can survive for the lifetime of the human body providing long lasting immunity to that particular antigen (Zimmerer et al., 2012). Upon secondary encounter with the same antigen, memory cells undergo robust clonal expansion resulting in the rapid elimination of that specific pathogen (Zielinski et al., 2011).

1.10 CD8 T cells

Effector CTLs provide vital protection against an array of intracellular bacterial and viral infections and are responsible for anti-tumour responses (Barber et al., 2006; Bos et al., 2012; Condotta et al., 2012). As MHC class I proteins exist on the surface of nearly every nucleated cell of the body, infected cells display pathogenic peptides derived from cytosolic proteins which can be recognised by CD8 T cells. The importance of CTL function is highlighted in a variety of disease settings such as chronic viral infections including hepatitis C virus (HCV), hepatitis B virus (HBV), human immunodeficiency virus (HIV) and malignancies including melanoma, ovarian, prostate and lung tumours which are a consequence of CTL dysfunction (Shin and Wherry, 2007; Matsuzaki et al., 2010; Chou et al., 2012; Fourcade et al., 2012; Prado-Garcia et al., 2012). Upon DC-mediated antigen stimulation and competent co-stimulation in the lymph node, activated antigen-specific CD8 T cells begin to secrete IL-2. This works in an autocrine fashion by binding to its high affinity IL-2R on the T cell surface, thus driving its exponential clonal expansion and functional differentiation into effector CTLs (Boyman and Sprent, 2012). CD8 T cell differentiation is highly dependent upon the transcriptional activity of T-box transcription factors, T box expressed in T cells (T-bet) and eomesodermin (EOMES) (Kaeche and Cui, 2012). The resultant CTLs then enter the blood stream and migrate to sites of infection where their effector responses generally peak at day 7 post-infection (Obar et al., 2008). Cytotoxic T lymphocytes target infected cells through numerous mechanisms including cellular-mediated cytotoxicity and cytokine secretion (Topham et al., 1997; La Gruta et al., 2004). Upon recognition of the antigen in conjunction with MHCI on the target cell, CTLs release cytolytic effector molecules including proteases granzyme A and B and the pore-forming perforin.

Perforin perturbs the membrane of the target cell allowing entry of granzyme A and B, the latter of which initiates a caspase-dependent apoptotic pathway, ultimately resulting in cell death (Lieberman, 2003). Additionally, CTLs release proinflammatory cytokines such as IFN γ and TNF α (La Gruta et al., 2004). Interferon- γ inhibits viral replication and promotes MHC I expression on infected cells thus increasing their probability of being targeted by activated CTLs. Tumour necrosis factor- α secretion activates innate immune cells and induces caspase-dependent cellular apoptosis via ligation with its cognate receptor TNFR-1 on the target cell (Murphy et al., 2012). Co-stimulation is critical for CTL effector function. Notably, CD28 signalling is associated with induction of IL-2 production. This subsequently enhances functional differentiation of CTLs through increasing granzyme and perforin expression (Janas et al., 2005; Williams et al., 2006).

CD8 TCR antigen affinity is an important factor that influences the CTL effector response. Generally, cross-presented self/tumour antigens with low avidity will be ignored by CD8 T cells, resulting in an absent effector response even in the presence of a viral infection (Bos and Sherman, 2010). CD8 T cells with a greater affinity for the tumour antigens, results in enhanced perforin and granzyme B expression, reduction in co-inhibitory receptor expression and induction of tumour irradiation (Bos et al., 2012). In these settings the requirement for enhanced CTL effector function is facilitated by the provision of CD4 T cell help (Bos and Sherman, 2010). CD4 T cells enhance CD8 T cell activation and differentiation via a variety of mechanisms including activated CD4 T cell secretion of IL-2 and IFN γ (Bos and Sherman, 2010). CD4 T cells can also directly interact with DCs via CD40:CD40L ligation. This enables the “licensing” of DCs and upregulation of DC CD80/86 co-stimulatory receptor expression, resulting in more efficient priming of CTL immune responses (Murphy et al., 2012).

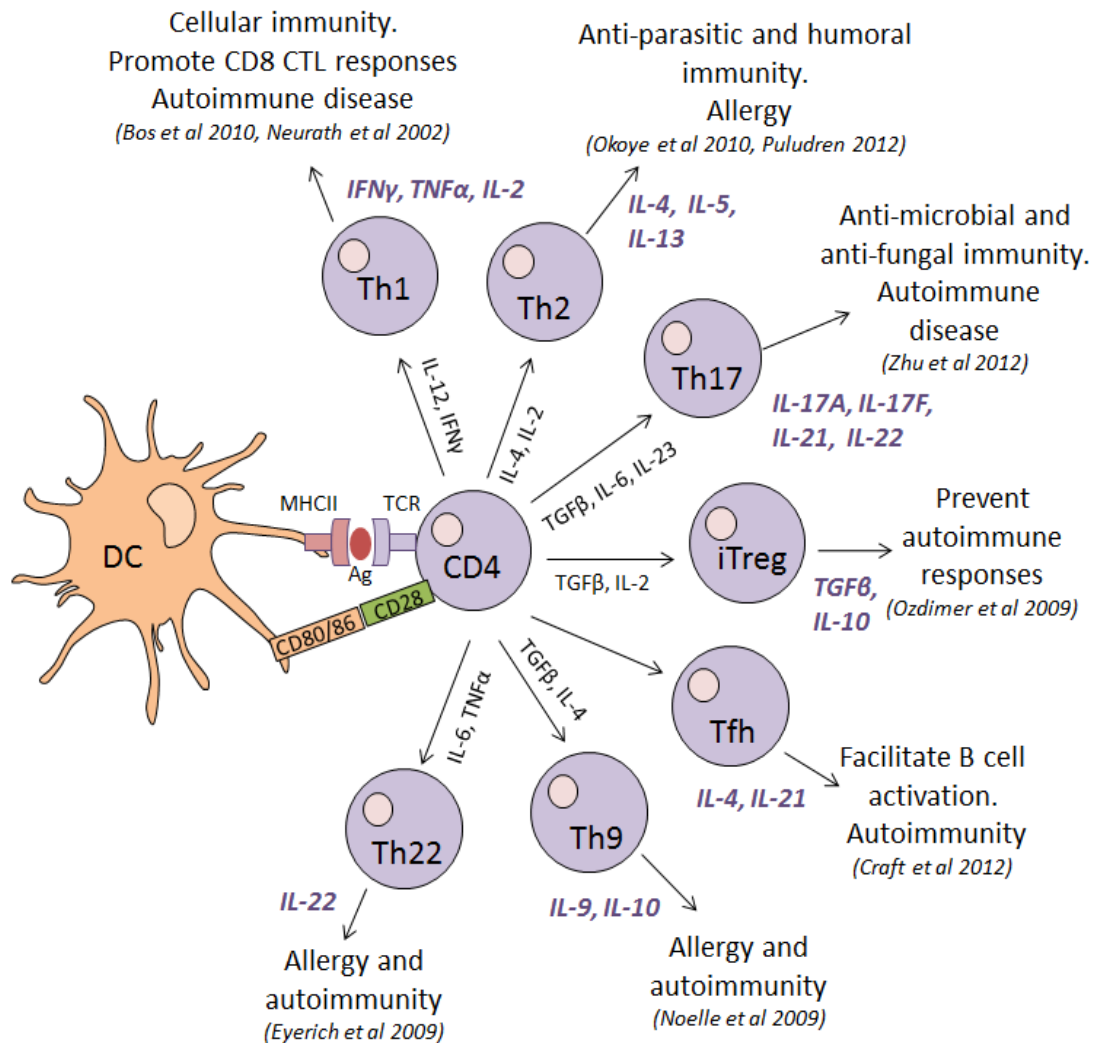


Figure 1.5. Dendritic cell-mediated CD4 T cell differentiation

Dendritic cells present extracellular-derived antigenic peptides in conjunction with MHCII molecules to naive CD4 T cells in secondary lymphoid organs. Upon TCR recognition of the specific antigen:MHCII complex and engagement of DC B7 CD80/86 with cognate T cell CD28 co-stimulatory molecules, this results in competent antigen-specific T cell activation, proliferation and differentiation. The cytokines present within the T cells' microenvironment encompass the vital tertiary signal necessary for instructing CD4 T cell differentiation into unique CD4 various subtypes: Th1, Th2, iTreg, Th17, Tfh, Th9 and Th22. Cytokines essential for inducing CD4 differentiation into the aforementioned subsets are shown in black. Each subtype is distinguished by their characteristic cytokine profile (shown in purple) and effector function. Dysregulation of CD4 differentiation is implicated in a variety of allergic disorders e.g. asthma and autoimmune diseases e.g. rheumatoid arthritis. Ag: antigen; DC: dendritic cell; IFN γ : interferon- γ ; IL: interleukin; iTreg: inducible T regulatory cell; MHCII: major histocompatibility complex class II molecule; TCR: T cell receptor; Tfh: CD4 T follicular helper cell; TGF β : transforming growth factor β ; Th: CD4 T helper cell; TNF α : tumour necrosis factor α .

1.11 CD4 T helper cells

CD4 T cells orchestrate complex immune responses against a myriad of pathogens. They can be categorised into a variety of subsets based on their distinctive cytokine profile, chemokine cell surface receptor pattern and immune function such as amplifying CD8 T cell immunity, facilitating B cell activation and preventing autoimmune responses (**Figure 1.5**). The earliest subtypes to be identified were the classical CD4 T helper 1 (Th1) and T helper 2 (Th2) cells (Mosmann et al., 1986). However, upon further investigation a variety of new distinct subsets were discovered including T regulatory T (Tregs), follicular helper T (Tfh) and the most recent T helper 17 (Th17), T helper 9 (Th9) cells and T helper 22 (Th22) cells (Chen et al., 2003; Harrington et al., 2005; Dardalhon et al., 2008; Eyerich et al., 2009; Craft, 2012). In contrast to CD8 T cells, CD4 T cells have greater plasticity in their differentiation which are not only dependent upon their cytokine milieu and the strength and duration of TCR signalling; but are also further regulated by a network of lineage-specific transcription factors and corresponding activation of specific signalling transducer and activator of transcription (STAT) proteins (Zhu and Paul, 2010). For the purposes of this introduction the main types of CD4 T cells (Th1, Th2, Th17 and Treg) and their dynamic interplay with one another are discussed in greater detail below.

1.11.1 Th1 cells

Effector Th1 cells play critical roles in mounting cell-mediated immune responses to intracellular viruses and pathogens such as *Listeria* (Hsieh et al., 1993). Effector Th1 cells are characterised by their secretion of IFN γ , TNF α and IL-2 as illustrated in **Figure 1.5** (Hsieh et al., 1993). Functional differentiation into Th1 is dependent upon exposure to proinflammatory cytokines e.g. IL-12 and IFN γ derived from innate immune cells (Hsieh et al., 1993; Lighvani et al., 2001). Notably, activated CD8⁺ DCs represent a major source of IL-12 and are thought to be the main drivers of Th1 derivation (Maldonado-Lopez et al., 1999). Recently, DC-derived IL-27 secretion has also been shown to be potent inducer of Th1 differentiation, playing a crucial role in the pathogenesis of skin inflammation associated with psoriasis

(Shibata et al., 2013). Induction of Th1 IFN γ secretion through DC-derived IL-12 secretion, activates innate immune cells including macrophages and neutrophils, enhancing their phagocytic function and microbial killing (Murphy et al., 2012); whilst Th1 IL-2 production promotes CD8 T cell activation and subsequent cytolytic capabilities (Janas et al., 2005). Multiple transcription factors play important roles in Th1 lineage development including the master transcription factor T-bet, Runx3, EOMES, NF- κ B and importantly STAT1 and STAT4 (Szabo et al., 2000; Lighvani et al., 2001; Malmberg et al., 2001; Djuretic et al., 2007; Yang et al., 2008; Kesarwani et al., 2012; Zhu et al., 2012). The strength of the TCR signal is important in determining Th1 lineage commitment. A strong TCR stimulation promotes Th1 differentiation whereas a weak TCR stimulation favours Th2 differentiation. (Tao et al., 1997). Increased Th1 differentiation and effector function has been implicated in the pathogenesis a variety of autoimmune diseases including Crohn's disease (Fuss et al., 1999; Neurath et al., 2002), MS (Frisullo et al., 2012), type 1 diabetes (Bluestone et al., 2010) and lupus nephritis (Ooi and Kitching, 2012).

1.11.2 Th2

Effector Th2 cells are central for the execution of humoral immunity in response to extracellular pathogens such as parasitic helminths and allergens (Pulendran and Artis, 2012). Effector Th2 cells are characterised by their production of IL-4, IL-5 and IL-13 (**Figure 1.5**). Interleukin-4 and IL-13 enhance B cell immune responses by promoting B cell immunoglobulin class switching from IgM towards IgE. This subsequently activates basophils and mast cells which secrete proinflammatory mediators which enhance the expulsion of helminth infection (Kool et al., 2012). Interleukin-5 enhances the activation and differentiation of eosinophils which are also involved in parasitic killing (Kool et al., 2012). Functional differentiation into Th2 is dependent upon naive T cell exposure to IL-2 and IL-4 (**Figure 1.5**). Interleukin-4 secretion is thought to be derived from innate immune cells such as eosinophils and basophils (Voehringer, 2013). Furthermore, thymic stromal lymphopoietin (TSLP) production by activated basophils, mast cells and epithelial cells at the site of infection can direct DCs to induce Th2 differentiation (Pulendran and Artis, 2012; Voehringer, 2013). Cd11c⁺ CD8⁻ DCs and monocyte-derived

inflammatory DCs play vital roles in Th2 differentiation during infection (Kool et al., 2012). Moreover, DCs matured under suboptimal or inflammatory conditions instruct the polarisation of CD4 T cells to Th2 phenotype (Pletinckx et al., 2011). Multiple transcription factors play an important role in Th2 lineage development including the master transcription factor GATA binding protein-3 (GATA-3), c-maf, growth factor independent-1 (Gfi-1), Interferon regulatory factor 4 (IRF4), Notch and importantly STAT proteins, STAT5 and STAT6 (Shimoda et al., 1996; Zhu et al., 2003; Pai et al., 2004; Fang et al., 2007; Luckheeram et al., 2012). The strength of the TCR signal is also important for determining Th2 differentiation. Lower peptide concentration induced activation of the TCR results in the increased production of CD4⁺ IL-4 producing cells (Yamane et al., 2005). Furthermore, weak TCR stimulation through the use of altered peptide ligands which possess a lower avidity for the receptor, results in the induction of Th2 cytokine production. This is also exclusively dependent upon CD28 co-stimulatory engagement (Tao et al., 1997). Weak TCR stimulation results in reduced ERK activation, favouring Th2 induction (Yamane et al., 2005). Although Th2 cells are vital for mounting humoral responses against helminth invasion, they are notorious for their role in triggering immune responses to innocuous environmental allergens, resulting in chronic inflammation associated with allergic diseases such as eczema, allergic rhinitis and asthma (Holgate, 2012; Murphy et al., 2012).

1.11.3 Th17 cells

In the past decade, a new CD4 subtype has been identified due to its distinguishing cytokine profile and transcriptional regulation atypical of the classical Th1/Th2 paradigm (Harrington et al., 2005; Ivanov et al., 2006). Furthermore, recognition of this new subtype was based on its observed pathophysiological role in experimental autoimmune models such as Experimental Autoimmune Encephalomyelitis (EAE), the animal model for human MS (Harrington et al., 2006). Previous studies concluded that the pathology exhibited in EAE was solely a result of a dominant Th1 phenotype (Liblau et al., 1995). The Th1 cytokine IL-12 composed of p40 and p35 subunits, was initially thought to be involved in Th1-driven pathology in EAE as IL-12p40^{-/-} mice were resistant to this autoimmune model (Gran et al., 2002).

However, further studies using IL-12p35^{-/-} mice showed a surprisingly enhanced susceptibility to EAE. This indicated that IL-12 was not involved but rather a structurally related cytokine to IL-12, IL-23 was responsible for promoting EAE (Gran et al., 2002). Furthermore, deficiency in IFN γ surprisingly exacerbated disease severity, ruling out Th1 cells as the main culprit behind the inflammatory-driven pathology associated with EAE (Ferber et al., 1996). Upon closer inspection, it was determined that a novel IL-17-producing CD4 effector T cell which is dependent on IL-23 rather than IL-12 for their expansion was in fact the actual offender in question (Harrington et al., 2005; Langrish et al., 2005; Komiyama et al., 2006). This was further clarified through the use of blocking IL-17 antibodies resulting in inhibition of EAE severity (Langrish et al., 2005; Komiyama et al., 2006). This effector subtype designated Th17 cells, play a pivotal role in orchestrating the host's defence against extracellular microbial and fungal pathogens, but also promote chronic inflammation associated with a variety of autoimmune diseases such as MS, Rheumatoid arthritis (RA), psoriasis and Systemic lupus erythematosus (SLE) (Zhu and Qian, 2012). Effector Th17 cells are characterised by their secretion of IL-17A, IL-17F, IL-21 and IL-22 (**Figure 1.5**) (Zhu et al., 2010). Th17-mediated cytokine functions include recruitment and expansion of neutrophils to sites of infection, activation of macrophages and DCs and the release of TNF α and IL-1 β , which collectively result in a heightened inflammatory response. (Zhu and Qian, 2012). Moreover, IL-17 promotes the activation and subsequent transformation of B cells into antibody secreting plasma cells (Zhu and Qian, 2012). Furthermore, IL-17 in conjunction with B cell activating factor (BAAF) has been shown to synergistically promote self-reactive B cell mediated pathogenesis in autoimmune SLE (Doreau et al., 2009). Functional differentiation into effector Th17 cells is governed by the secretion of TGF β , IL-6 and IL-23 from DCs (**Figure 1.5**) (Mangan et al., 2006; Veldhoen et al., 2006). Although IL-23 has been shown to be dispensable for the development of Th17 cells *in vitro*, it is essential for their phenotypic stability, expansion and effector function against microbial infection *in vivo* (Oppmann et al., 2000; Mangan et al., 2006; McGeachy et al., 2007). Proinflammatory cytokines IL-1 β and TNF α have also been shown to promote Th17 differentiation (Veldhoen et al., 2006). Co-stimulatory receptor engagement of

CD28, CD40L and ICOS are also necessary for Th17 generation (Park et al., 2005b; Huang et al., 2012). Multiple transcription factors play an important role in Th17 lineage development including the master transcription factor retinoic acid-related orphan receptor- γ t (ROR γ t or RORC2 human equivalent), nuclear aryl hydrocarbon receptor (AHR), IRF4, B cell-Activating Transcription Factor (BTAF), Runx1 and STAT3 (Ivanov et al., 2006; Mathur et al., 2007; Huber et al., 2008; Veldhoen et al., 2009; Zhu et al., 2010), Expression of ROR γ t is obligatory for Th17 development which is upregulated by TGF β and IL-6 (Ivanov et al., 2006; Burgler et al., 2010). It has been shown that DCs are the main drivers of the pathogenic Th17 phenotype (Veldhoen et al., 2006; Huang et al., 2012; Shi et al., 2012). Furthermore, DC signalling via the p38 MAPK pathway is necessary for the promotion of Th17 differentiation and subsequent pathogenesis of EAE as highlighted in p38 MAPK deficient mice (Huang et al., 2012).

1.11.4 T regulatory cells (Treg)

T regulatory cells are specialised suppressors of the immune responses. They are responsible for the maintenance of immune homeostasis, elimination of self-reactive T cells and subsequent prevention of autoimmune responses (Sakaguchi et al., 2008). Reduced numbers of Tregs are observed in a variety of autoimmune diseases such as MS, RA and SLE revealing the fundamental role of this subset in maintaining self-tolerance (Ozdemir et al., 2009). There are two main types of Tregs; natural occurring CD4⁺CD25⁺Foxp3⁺ (nTregs) and inducible Tregs (iTregs) which can be differentiated from CD4⁺CD25⁻Foxp3⁻ T cells in the periphery (Ozdemir et al., 2009). The former are derived from the thymus and are positively selected based on strong interactions with thymic stromal cells (Sakaguchi et al., 2008). Natural occurring Tregs constitutively express CD25 and the master transcription factor Foxp3, the latter of which is vital for their development and suppressive function (Burchill et al., 2007). Natural occurring Tregs elicit their suppressive function in response to extremely low concentrations of antigen in comparison to concentrations required to activate naive CD4 T cells (Takahashi et al., 1998). Suppression is achieved via a variety of contact-dependent mechanisms such as inhibiting IL-2 formation by responder T cells (Takahashi et al., 1998), targeted

killing of CD8 and NK T cells (Cao et al., 2007) and directly outcompeting responder T cell interactions with APCs (Yamaguchi et al., 2011). Additionally, the co-inhibitory receptor Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) which is highly expressed on nTregs, further enables their suppressive activity. (Wing et al., 2008). Within the appropriate context, peripheral $CD4^+CD25^-Foxp3^-$ T cells can differentiate into $CD4^+CD25^+Foxp3^+$ T cells which acquire the unique characteristics of nTregs; these polarised effector CD4 T cells are termed iTregs (Chen et al., 2003). Upon antigen stimulation and $TGF\beta$ exposure, Foxp3 is upregulated in peripheral $CD4^+$ T cells, resulting in their conversion to $CD4^+CD25^+Foxp3^+$ T cells (**Figure 1.5**) (Chen et al., 2003; Zheng et al., 2004). Inducible Tregs secrete the immunosuppressive cytokines IL-10 and $TGF\beta$ which dampen inflammatory responses, but can also further instruct the differentiation of peripheral CD4 T cells to become suppressor T cells (**Figure 1.5**) (Zheng et al., 2004). Interleukin-10 secretion can in turn induce tolerogenic DCs, further dampening immune responses (Yamaguchi et al., 2011). Dendritic cells play a pivotal role in the differentiation of peripheral iTregs (Yamazaki et al., 2007). Splenic steady-state DCs presenting low antigen concentrations in the presence of exogenous $TGF\beta$, results in the induction of Foxp3 in peripheral $CD4^+$ T cells (Yamazaki et al., 2007). The gut which is constantly exposed to a myriad of infectious and innocuous microbes is a prime location for DC-mediated iTreg differentiation. Within this region, tolerance to harmless antigens is necessary to avoid regular induction of aberrant immune responses (Scott et al., 2011). In support of this, it has been shown that tolerogenic mesenteric lymph node (MLN) DCs have an enhanced ability to convert CD4 T cells into iTregs in the presence of $TGF\beta$ and IL-2 in comparison to skin draining LN DCs (Yamazaki et al., 2007).

1.11.5 Other effector CD4 T cell subtypes: Tfh, Th9, Th22

T follicular helper (Tfh) cells are a B cell helper T cell subset responsible for the facilitation of B cell survival, differentiation and immune function (Craft, 2012). They are localised in B cell-rich germinal centres, where upon activation they form contacts with B cells, promoting B cell maturation, immunoglobulin antibody class switching to IgE and subsequent plasma cell antibody production (Craft, 2012). They

secrete IL-4 and IL-21 which are essential for B cell help and are transcriptionally regulated by B cell lymphoma-6 (BCL6) (Craft, 2012). Dysregulation of Tfh cells have been shown to play a detrimental role in B cell-mediated autoimmune responses observed in SLE (Craft, 2012).

Another subset of T cells namely Th9 (once thought to be Th2 cells) are distinctive in their cytokine production of IL-9 and IL-10 and transcriptional regulation via PU.1, IRF4 and GATA-3 (Dardalhon et al., 2008; Noelle and Nowak, 2010). Furthermore STAT6 is necessary for their development (Noelle and Nowak, 2010). Upon antigen stimulation naive CD4 T cells differentiate into Th9 cells in the presence of IL-4 and TGF β (Dardalhon et al., 2008). Effector Th9 cells have been shown to promote tissue inflammation in murine colitis model and it is becoming increasingly apparent that this subset plays a key pathogenic role in allergic and autoimmune diseases (Dardalhon et al., 2008; Noelle and Nowak, 2010).

In recent years accumulating evidence of a novel CD4 specific lineage designated Th22 cells has emerged, that can secrete IL-22 independently of IL-17 and is present in a variety of allergic skin disorders (Eyerich et al., 2009). Furthermore, these cells express chemokine receptors CCR6, CCR4 and CCR10 (Duhon et al., 2009). Effector Th22 cells have been shown to be localised in the skin epidermis in patients with psoriasis, atopic eczema and allergic contact dermatitis. They are suggested to be involved in epidermal tissue remodelling and hyperplasia associated with these skin diseases (Eyerich et al., 2009). Differentiation into Th22 cells is dependent upon the presence of IL-6 and TNF α within the T cell microenvironment (Duhon et al., 2009). Furthermore, mature pDCs and not cDCs can efficiently produce high levels of IL-6 and TNF α thus are involved in promoting Th22 differentiation (Duhon et al., 2009).

1.12 Interplay between the CD4 subsets

In addition to the suppressive actions of Tregs in regulating immune function and immunological tolerance, the varying CD4 populations themselves control the development and differentiation of one another, sustaining a quintessential balance between the unique CD4 T cell subtypes and ultimately maintaining immune homeostasis. It is also worthy to note that CD4 subsets are not terminally

differentiated in that if exposed to different optimal polarising conditions, they can acquire the characteristics of other CD4 subsets (Veldhoen et al., 2006; Lee et al., 2009). The relationships between the differing subtypes are discussed below.

1.12.1 Th1/Th2 cross talk

Numerous studies have dissected the integral relationship between Th1 and Th2 cells. It is obvious that Th1 cells counter regulate the differentiation of Th2 cells and vice versa. Moreover, neutralising antibodies for IL-4 and IFN γ , or IL-12 lineage-specific cytokines are routinely used in *in vitro* studies in optimal polarising conditions for Th1 and Th2 differentiation, respectively (Nurieva et al., 2007). Indeed, T-bet expression results in the downregulation of GATA-3, the converse also true (Usui et al., 2006; Gu et al., 2012). Furthermore, overexpression of GATA-3 and T-bet in CD4 T cells results in the differentiation of Th2 and Th1 cells, correspondingly (Szabo et al., 2000; Usui et al., 2003; Usui et al., 2006). Additionally, GATA-3 and T-bet inhibit the activity of the lineage specific STAT4 and STAT5, respectively (Usui et al., 2003; Shatynski et al., 2012). Reciprocal regulation of Th1/Th2 cells has been observed in other lineage specific transcription factors such as c-maf mediated IFN γ suppression (Ho et al., 1998), Gfi-1 negative regulation of Th1 differentiation (Zhu et al., 2009), Th2 GATA-3 regulation of Runx3 (Djuretic et al., 2007) and Runx3-mediated suppression of IL-4 (Djuretic et al., 2007). Disease settings in which the Th1/Th2 balance is disrupted permitting a dominant Th2 phenotype, as seen in allergic disorders e.g asthma and eczema highlights the importance of equilibrium between these crucial CD4 effector populations in immune responses (Holgate, 2012).

1.12.2 Th1/Th2/th17 cross talk

In addition to the Th1/Th2 paradigm, it has become increasingly apparent that cross regulation exists between Th1/Th2 and Th17 subtypes. It has been shown that IFN γ and IL-4 both inhibit Th17 development (Harrington et al., 2005). Generally *in vitro* treatment with neutralising antibodies for IL-4 and IFN γ in conjunction with Th17 inducing cytokines is the typical approach employed for induction of optimal Th17 differentiation (Harrington et al., 2005; Park et al., 2005b; Nurieva et al., 2007).

Both STAT1 and T-bet have been shown to inhibit CD4⁺IL-17⁺ producing cells (Harrington et al., 2005). Furthermore, GATA-3 overexpression results in STAT3 and ROR γ t inhibition and subsequent impairment of Th17 differentiation (van Hamburg et al., 2008). Moreover, increased CD4⁺IL-17-producing cells are observed in STAT5 deficient mice (Laurence et al., 2007). Th2 transcription factors c-maf and Gfi-1 has also been shown to negatively regulate Th17 differentiation, the latter of which also impairs iTreg differentiation in the presence of TGF β (Park et al., 2005b; Zhu et al., 2009). Both IL-23 and IL-21 have been shown to inhibit the expression of T-bet (Nurieva et al., 2007; Mus et al., 2010). Interleukin-27 also inhibits ROR γ t expression and subsequent Th17 differentiation in murine naive CD4 T cells (El-behi et al., 2009). Donor CD4 T cells derived from IFN γ deficient mice exhibit enhanced Th2 and Th17 differentiation in GVHD murine model, further emphasising the cross regulation between the various subtypes (Yi et al., 2009).

1.12.3 Treg/Th17 cross talk

Not surprisingly, an integral link between Treg and Th17 also exists in the immune system as they both require TGF β in different contexts for their development. Under normal conditions where TGF β is present but IL-6 limiting, the immunosuppressive Treg population prevails over the Th17 population. However upon inflammatory conditions whereby both cytokines are present, the Treg differentiation is hindered whilst allowing the proinflammatory Th17 phenotype to dominate (Bettelli et al., 2006; Veldhoen et al., 2006; Burgler et al., 2010; Laurence et al., 2012). Indeed, the lineage specific transcription factors Foxp3 (Treg) and ROR γ t/RORC (Th17) are known to antagonise the expression of one another; thus Tregs and Th17 cells express low levels of ROR γ t and Foxp3, respectively (Bettelli et al., 2006; Mangan et al., 2006; Burgler et al., 2010). Th17 cytokines such as IL-6, IL-23 and IL-21 have also been shown to suppress Foxp3 expression (Bettelli et al., 2006; Nurieva et al., 2007; Huber et al., 2008; Mus et al., 2010; Laurence et al., 2012). Similarly, while essential for Treg development, IL-2 potentially inhibits Th17 differentiation (Laurence et al., 2012). The gut localised CD103⁺ DCs release retinoic acid that supports iTreg differentiation, while hampering Th17 development (Scott et al., 2011). Moreover, under inflammatory conditions associated with GVHD,

STAT3 inhibits Foxp3 expression and subsequent iTreg differentiation whilst promoting Th17 development (Laurence et al., 2012).

1.13 Redox and its role in immune cell function

Cellular redox is a state arising from the combined contribution of oxidising and reducing elements. It impacts on numerous physiological processes involved in cell activation, proliferation, differentiation, cell survival and apoptosis (Valko et al., 2007). The maintenance of cellular redox homeostasis is imperative for proper immune cell functioning (Kesarwani et al., 2012). Cellular redox homeostasis is achieved via an equilibrium between oxidising agents such as electrophiles, reactive oxygen species (ROS), chemical, drugs and their respective metabolites, and reducing systems including enzymatic anti-oxidants e.g. Superoxide dismutase (SOD), ROS scavenging vitamins and the non-protein thiol glutathione (GSH) (Nathan and Cunningham-Bussel, 2013). During cellular metabolic processes, highly reactive oxygen-derived free radicals namely ROS are produced as by-products (Valko et al., 2007). Mitochondrial oxidative metabolism, in which the consumption of oxygen and subsequent oxidation of NADH mediated by NADPH oxidase (NOX) enzyme, produces the ROS superoxide anion ($O_2^{\cdot-}$) (Ma, 2010). Other ROS include hydroxyl radical ($\cdot OH$), peroxy radical (RO_2^{\cdot}) and the non-radical hydrogen peroxide (H_2O_2) (Ma, 2010). Reactive oxygen species can also be generated through the induction of other enzymes including xanthine oxidase (XO), lipoxygenases, and phase I cytochrome P450s (CYP450) drug metabolising enzymes. Other endogenous sources of ROS include heme groups, metal storage proteins and free iron and copper ions (Yu, 1994; Valko et al., 2007; Imlay, 2008; Ma, 2010; Nathan and Cunningham-Bussel, 2013). Moreover, environmental factors such as diesel exhaust fumes, smoking, metal exposure, Ultraviolet (UV) radiation and xenobiotic metabolism all influence ROS production (Nathan and Cunningham-Bussel, 2013). Due to their instability and heightened reactivity within the body, ROS are generally thought of as dangerous by-products owing to their capacity to induce cellular damage if housekeeping anti-oxidant defence systems are limiting (Nathan and Cunningham-Bussel, 2013). Nonetheless, it is evident that transient ROS production plays a positive physiological role within the body (Nathan and Cunningham-Bussel,

2013). ROS operate as important messengers of signal transduction. This is through the oxidative modification of kinases and phosphatases which are present in many signalling pathways including MAPK and NF- κ B. Therefore, ROS are crucial for the proper functioning of both the innate and adaptive arms of the immune system (Ryan et al., 2004; Matsuzawa et al., 2005; Ma, 2010; Nathan and Cunningham-Bussel, 2013). With reference to innate immune cell function, activated neutrophils and macrophages are reliant upon the generation of ROS via oxidative metabolism in order to efficiently engulf and destroy noxious pathogens, a process that is highly dependent upon their expression of NOX (Morel et al., 1991). ROS are necessary for efficient TLR signalling in innate immune cells including monocytes and DCs. This results in the elevation of downstream p38 MAPK and NF- κ B activation, mediating enhanced proinflammatory cytokine production e.g. IL-1 β , IL-8 and IL-6 which in turn shape the adaptive immune response (Ryan et al., 2004; Matsuzawa et al., 2005). The differentiation of DCs from haematopoietic progenitor cells is highly dependent on GM-CSF-induced mitochondrial ROS generation (Del Prete et al., 2008; Sheng et al., 2010). Moreover, ROS promotes LPS-induced DC maturation through the up regulation of CD86 subsequently enhancing DC-mediated CD4 T cell proliferation and effector function (Matsue et al., 2003). In contrast, O₂⁻ can induce DC maturation in the absence of LPS mediated TLR signalling, through enhanced NF- κ B activation associated with increased CD86 expression and subsequent increased DC-mediated antigen presentation ((Kantengwa et al., 2003). ROS have also been shown to potentiate TLR4-mediated DC cytokine production of IL-12 in response to the cysteine protease papain, resulting in downregulation of Th1 differentiation (Tang et al., 2010). Therefore, ROS generation is important for maintaining DC mediated CD4 T cell differentiation towards a Th2 phenotype.

From an adaptive T cell perspective, mild ROS generation is necessary for optimal TCR signalling and T cell activation resulting in enhanced IL-2 production (Lu et al., 2007). Furthermore, mitochondrial generation of ROS in T cells induces the increased cell surface expression of the T cell activation markers CD25 and CD69 (Sena et al., 2013). ROS are also required for CD4 antigen-specific TCR-dependent proliferation and effector function *in vivo* and is associated with appropriate IL-4, IL-

5 and IL-13 production in response to allergen (Sena et al., 2013). ROS are necessary for mediating antigen-specific CD8 T cell activation, expansion and increased effector IFN γ production in response to *Listeria monocytogenes* infection (Sena et al., 2013). T cell NOX2-dependent ROS generation is essential for the induction of Th2 differentiation associated with increased STAT5 activation and GATA-3 expression (Shatynski et al., 2012). This suggests that similar to DC-mediated ROS generation, T cell-mediated ROS generation during naïve T cell activation is also essential for modulating CD4 T cell differentiation enabling Th2 dominance over Th1 (Shatynski et al., 2012).

Intracellular ROS accumulation is normally counterbalanced by an array of intricate anti-oxidant defence mechanisms, which effectively detoxify hazardous free radicals, enabling their safe removal from the body. The cellular tripeptide glutathione (γ -L-glutamyl-L-cysteinyl-glycine) (GSH) is the main reductant/antioxidant found ubiquitously throughout the body (Lu, 2009). This non protein thiol is found mainly in the cytoplasm in its reduced state where it operates as a cellular redox buffer (Lu, 2009). It has many physiological functions including the detoxification of oxidising drugs and their respective metabolites, and the decomposition of H₂O₂ into water. Furthermore, GSH can participate in thiol-disulphide exchange reactions with proteins, thus protecting protein thiol groups from ROS or electrophilic-mediated oxidation (Talalay et al., 2003; Zhang et al., 2011). The specific role of GSH in immune cell function is exemplified in **Table 1.3**. Glutathione biosynthesis proceeds via a two-step mechanism. The initial rate limiting step involves the binding of L-glutamate to L-cysteine to form γ -glutamyl-L-cysteine catalysed by enzyme glutamate cysteine ligase (GCL) which is composed of catalytic (GCLc) and regulatory modifier (GCLm) subunits (Lu, 2009). The final step involves the enzymatic GSH synthase-mediated generation of GSH from γ -glutamyl-L-cysteine and L-glycine (Lu et al 2009). One of the drivers of GSH biosynthesis is the redox sensitive transcription factor Nuclear factor-erythroid 2 (NF-E2)–related factor 2 (Nrf2) which is discussed in section **1.15**.

Cell type	Immune function	Reference
Dendritic cells	<ul style="list-style-type: none"> Regulates DC-mediated antigen specific T cell proliferation Modulates differentiation from monocyte precursors Controls DC-mediated Th1/Th2 differentiation towards Th1 dominance through modulation of DC IL-12 and IFNγ secretion Enhances IL-27 and IL-12 DC secretion resulting in Th1 dominance Limits DC-mediated T cell secretion of IL-13 in the presence of Th2-inducing cytokine TSLP Provides DC-mediated extracellular reducing environment for optimal T cell proliferation 	(Peterson et al., 1998; Kuppner et al., 2003; D'Angelo et al., 2010; Yan et al., 2010; Kamide et al., 2011)
Macrophages	<ul style="list-style-type: none"> Modulates LPS-induced IL-12 secretion through JNK and p38 MAPK regulation Potentiates LPS-induced IL-6 secretion Maintains macrophage-mediated Th1/Th2 balance 	(Dobashi et al., 2001; Murata et al., 2002a; Murata et al., 2002b; Utsugi et al., 2003)
NK cell	<ul style="list-style-type: none"> Promotes cytolytic capacity in response to <i>Mycobacterium tuberculosis</i> infection 	(Millman et al., 2008)
T cell	<ul style="list-style-type: none"> Regulates T cell-activated induced cell death Provides reducing environment for DNA synthesis within the T cell and progression through the cell cycle Tregs inhibit Teff proliferation through decreasing DC intracellular GSH levels. This limits extracellular cysteine availability for reducing environment for Teff activation. Tregs prevent redistribution of intracellular GSH from nucleus to cytoplasm within Teff which is necessary for Teff proliferation. 	(Messina and Lawrence, 1989; Suthanthiran et al., 1990; Chiba et al., 1996; Lee et al., 2010) (Yan et al., 2010)

Table 1.3 The role of the antioxidant glutathione in the maintenance of immune cell function

DC: dendritic cell; GSH: glutathione; IL-: interleukin-; JNK: c-Jun N-terminal kinase; LPS: lipopolysaccharide; p38: p38 mitogen-activated protein kinase (MAPK); Teff: CD4 effector T cell; Th: CD4 helper T cell; TSLP: thymic stromal lymphopoietin

1.14 Oxidative stress and immune cell function

Sustained oxidative stimuli e.g. excessive ROS production, leads to a depletion of cellular GSH. This overpowers the anti-oxidant defence systems and disrupts the cellular redox equilibrium by shifting it towards an oxidative state, ultimately resulting in oxidative stress (Kesarwani et al., 2012). Moreover, defects in the detoxification machinery itself can often lead to a similar outcome (Case et al., 2011). Prolonged oxidative stress can lead to cellular dysfunction, apoptosis or necrosis (Durackova, 2010). Increased ROS can cause the aberrant oxidation of proteins resulting in their modification, denaturation and subsequent proteasomal degradation (Yu, 1994). Furthermore, it enhances lipid peroxidation ensuing loss of cellular membrane integrity. Importantly, it attacks nucleic acids resulting in double stranded DNA breaks or base mutations (Yu, 1994).

Oxidative stress has a major impact on the appropriate functioning of DCs. It has been shown to induce DC maturation as typified by enhanced CD80, CD86 and MHCII expression (Traidl-Hoffmann et al., 2005; Csillag et al., 2010; Wang et al., 2011). Conversely, oxidative stress can perturb LPS-induced DC maturation through down regulation of MHCII, CD86, CD54 (Chan et al., 2006), CD40, and lymphoid homing receptor CCR7 (Vassallo et al., 2005). Similarly, oxidative stress can either enhance (Traidl-Hoffmann et al., 2005; Wang et al., 2011) or potentiate (Vassallo et al., 2005) DC-mediated T cell proliferation. This highlights the variable role of oxidative stress in the DC maturation process and subsequent DC-mediated T cell activation. Consistently, oxidative stress influences DC regulation of Th1/Th2 polarisation mediating a shift towards the Th2 phenotype (Ohtani et al., 2005; Traidl-Hoffmann et al., 2005; Vassallo et al., 2005; Chan et al., 2006; Csillag et al., 2010).

With reference to T cell function, excess mitochondrial production of superoxide at the stage of Lck activation in developing T cells, enhances thymocyte apoptosis resulting in a reduction of mature CD4 and CD8 T cell populations in the thymus and in peripheral lymphoid organs. Furthermore, the resultant peripheral CD4 and CD8 T cells exhibit an enhanced activation state associated with increased CD44

expression (Case et al., 2011). Similarly, a marked downregulation of CD62L in naïve CD4 T cells is observed upon oxidative stress exposure (Foster et al., 2013). Increased ROS levels have been associated with T cell hyporesponsiveness due to dysregulated TCR signalling (Malmberg et al., 2001; Gringhuis et al., 2002; Cemerski et al., 2003). Excessive ROS production reduces CD8 T cell survival and IFN γ production in response to viral infection, which is associated with delayed viral clearance (Lang et al., 2013). In line with oxidative stress-mediated DC dysfunction, chronic oxidative stress has been known to influence CD4 T cell functional differentiation through disruption of the Th1/Th2 balance. This is evident in a variety of Th2 driven inflammatory allergic settings such as asthma and atopic skin diseases (Dieckhoff et al., 2005; Holgate, 2012). Oxidative stress-mediated reduction in Th1 effector function in AD T cells was associated with decreased nuclear translocation of NF- κ B (Malmberg et al., 2001; Dieckhoff et al., 2005). Oxidative stress also influences Th1/Th17 polarisation skewing towards the Th17 phenotype within a collagen induced arthritis murine model (Zhi et al., 2012). Furthermore oxidative stress mediated an increased pulmonary Th17 phenotype in a murine model of asthma (Wang et al., 2011). Oxidative stress is counterbalanced by antioxidant and cytoprotective defence responses which are controlled by the transcriptional activity of Nrf2.

1.15 Nrf2: Master regulator of the anti-oxidant defence response

The induction of cytoprotective and anti-oxidant defence systems in response to electrophilic and oxidative insult, and ultimate maintenance of cellular redox is governed by the transcriptional activity of the cap'n'collar (CNC) basic region leucine zipper (bZip) transcription factor Nrf2 (Copple et al., 2010). Nrf2 is present in many tissues including heart, pancreas, muscle and particularly in tissues that are continually subjected to environmental toxins such as the lungs, gastrointestinal (GI) tract and skin (Moi et al., 1994; Aleksunes and Manautou, 2007). Furthermore, Nrf2 is highly concentrated in drug metabolising regions such as liver and kidney tissues, where it plays an essential role in the metabolism and detoxification of xenobiotics and their respective reactive metabolites (Moi et al., 1994; Ma and He, 2012). Under resting conditions Nrf2 is sequestered in the cytosol by the repressor

protein Kelch-like ECH-associated protein 1 (Keap-1) (Zipper and Mulcahy, 2002). Keap-1 targets Nrf2 for polyubiquitination and rapid degradation by the proteasome (**Figure 1.6**). However under oxidative stress conditions, cellular levels of GSH are markedly depleted, resulting in the oxidation and modification of the Keap-1 protein. This induces a conformational change in Keap-1 disabling it from directing Nrf2 for proteasomal degradation (Coppo, 2012). As a result, Nrf2 saturates Keap-1. Once Keap-1 is saturated, newly synthesised Nrf2 is available to freely translocate into the nucleus (**Figure 1.6**). Within the nucleus, Nrf2 binds to the *cis*-acting element, namely antioxidant response element (ARE), present in the promoter regions of an array of Nrf2-regulated cytoprotective and antioxidant genes (Itoh et al., 1997). This results in the induction of transcription of genes involved in GSH synthesis e.g. GCL, enzymatic antioxidants e.g. SOD, and detoxification enzymes e.g. NQO1 (**Figure 1.6**) (Coppo, 2012). Consequently, GSH cellular content is increased resulting in cellular protection and maintenance of redox homeostasis. The main function and immunological relevance of the Nrf2-regulated cytoprotective and antioxidant defence proteins is depicted in **Table 1.4**.

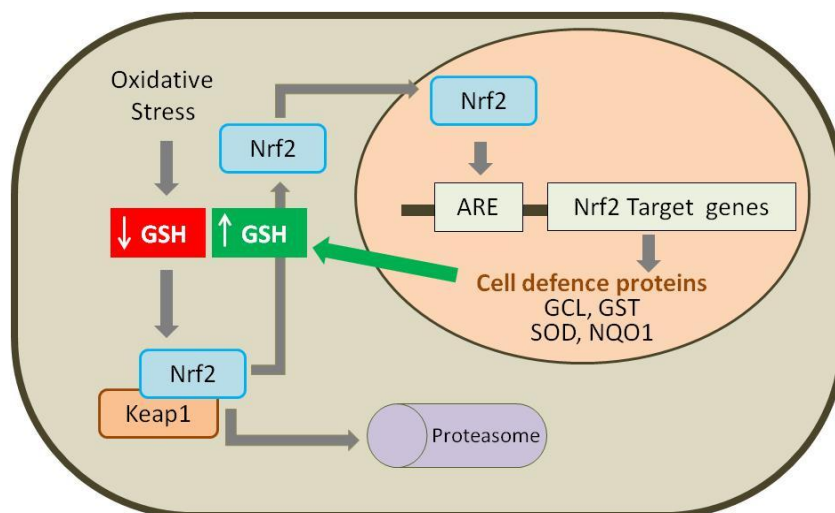


Figure 1.6 Schematic diagram of the Nrf2-Keap-1 pathway

Nrf2 is sequestered in the cytosol by Keap-1, which directs Nrf2 for proteasomal degradation. Upon oxidative insult, Nrf2 is liberated from Keap-1 enabling Nrf2 to accumulate within the nucleus and transactivate cytoprotective genes. ARE: antioxidant response element; GCL: glutamate cysteine ligase; GSH: glutathione; GST: glutathione S transferase; Keap-1: Kelch-like ECH-associated protein 1 NQO1: NAD(P)H: quinone oxidoreductase; Nrf2: NF-E2-related factor 2; SOD: superoxide dismutase.

Protein	Function	Immune relevance	References
Glutamate cysteine ligase (GCL): Catalytic subunit (GCLc) Regulatory modifier subunit (GCLm)	Rate limiting enzyme in GSH synthesis. Catalyses the ligation of glutamate with cysteine to form γ -glutamyl-L-cysteine	<ul style="list-style-type: none"> Regulate neutrophil infiltration and inflammatory cytokine production in response to air pollutant in the lung Important role in senescence, mellitus diabetes and cancer all associated with immune dysregulation 	(Lu, 2009; Weldy et al., 2011; Zhang et al., 2011)
Glutathione peroxidase (GPX)	Catalyses the reduction of H_2O_2 to H_2O using GSH as a substrate	<ul style="list-style-type: none"> Protects host against viral-induced pathogenesis Regulates T cell intracellular ROS levels in response to CD3 and CD28 stimulation Controls TCR-mediated T cell proliferation and IL-2 production Important for maintenance of CD4 differentiation into Th1, Th2 and Th17 cells 	(Beck et al., 1998; Zhang et al., 2011) (Beck et al 1998)(Won et al., 2010)
Glutathione reductase (GSR)	Catalyses the reduction of glutathione disulphide (GSSG) into GSH with NADPH utilisation	<ul style="list-style-type: none"> Required for mounting innate immune response to bacterial infection Regulates phagocytosis, respiratory burst and bactericidal activity in neutrophils 	(Yan et al., 2012; Lu, 2013)
Glutathione-S-transferase (GST)	Catalyses the conjugation of GSH to a variety of electrophilic compounds	<ul style="list-style-type: none"> Regulates proinflammatory mediator expression in skin cancer 	(Henderson et al., 2011; Lu, 2013)

Heme-oxygenase 1 (HO-1)	Catabolizes cellular heme to biliverdin, carbon monoxide and free iron	<ul style="list-style-type: none"> Regulates splenic CD4:CD8 ratio Modulates Th1, Th2 and Th17 differentiation Controls LPS-induced monocyte IL-1β and TNFα production Required for APC-mediated Treg immunosuppressive function Important for macrophage secretion of IFNβ and anti-viral immunity Protects against EAE and modulates Th17 polarisation 	(Poss and Tonegawa, 1997; Kapturczak et al., 2004; George et al., 2008; Rushworth et al., 2008; Xia et al., 2008; Tzima et al., 2009)
NAD(P)H:quinine oxidoreductase 1 (NQO1)	Catalyze two-electron metabolic reduction and detoxification of quinones and derivatives	<ul style="list-style-type: none"> Controls LPS-induced monocyte IL-1β and TNFα production Regulates B cell mediated humoral immunity Important for maintenance of T cell tolerance 	(Iskander et al., 2006; Rushworth et al., 2008)
Peroxiredoxin 1 (PRX1)	Catalyses the decomposition of H ₂ O ₂ into H ₂ O and O ₂	<ul style="list-style-type: none"> Implicated in regulation of T cell anti-viral immunity Potentiates NK cell cytotoxicity Regulates Th1/Th2 balance in response to allergen challenge 	(Zhang et al., 2011; Ishii et al., 2012)
Superoxide dismutase (SOD)	Catalyzes the conversion of superoxide radicals into H ₂ O ₂ and O ₂	<ul style="list-style-type: none"> Involved in T cell development in the thymus and subsequent T cell populations in lymphoid organs Necessary for elicitation of adaptive immune response to viral infection 	(Yu, 1994; Case et al., 2011)

Thioredoxin (TRX)	Catalyses the reduction of disulphide bonds to sulphhydryls Acts as a ROS scavenger for singlet oxygen and hydroxyl radicals	<ul style="list-style-type: none"> Regulates neutrophil migration and adhesion to endothelial cells via modulation of MCP-1 and RANTES in response to LPS Regulates Th1 cytokine production 	(Das and Das, 2000; Nakamura et al., 2001; Kang et al., 2008)
Thioredoxin reductase (TRXR)	Catalyses the reduction of oxidised TRX into TRX with NAPH utilisation	<ul style="list-style-type: none"> Essential for VSIG₄ expression in macrophages. VSIG₄ expression in APC is important for the maintenance of T cell reactivity 	(Nakamura et al., 2005; Vogt et al., 2006; Carlson et al., 2011)

Table 1.4. Cytoprotective and antioxidant proteins encoded by Nrf2-related genes and their immunological relevance.

The immunological evidence is based on findings that use knockout mice and/or knockdown siRNA methods to delineate the precise role of the relevant proteins in immune cell function. VSIG₄ is a member of the B7 protein family functioning as an APC co-inhibitory receptor (in a similar way to CTL4-4) involved in the negative regulation of T cell activation. APC: antigen-presenting cell; EAE: experimental autoimmune encephalomyelitis; GSH: glutathione; H₂O: water; H₂O₂: hydrogen peroxide; IFN β : interferon- β ; IL-: interleukin-; MCP-1: monocyte chemotactic protein-1 also known as chemokine (C-C motif) ligand-2 (CCL2); NADPH: Nicotinamide adenine dinucleotide phosphate; O₂: oxygen; RANTES: Regulated on Activation, Normal T cell Expressed and Secreted also known as CCL5; ROS: reactive oxygen species; Th: CD4 T helper cell; TNF α : tumour necrosis factor- α ; Treg: T regulatory cell; VSIG₄: V-set and immunoglobulin domain containing 4. *Adapted from (Copple et al., 2010)*

1.16 The role of Nrf2 in the maintenance of redox homeostasis

The role of Nrf2 in the regulation of cellular defence in response to oxidative stress, protection against oxidative stress-induced tissue injury and disease has been demonstrated in investigations where the Nrf2 pathway has been perturbed. This is mainly through the use of Nrf2 deficient mice, genetic manipulation of Nrf2 through siRNA knockdown in murine and human primary cells, and Nrf2 amplification studies using Keap-1 siRNA and Nrf2-inducing drugs such as CDDO-Me (Rangasamy et al., 2005; Thimmulappa et al., 2006b; Thimmulappa et al., 2007; Yates et al.,

2007; Williams et al., 2008; Reddy et al., 2009a; Williamson et al., 2012; Cui et al., 2013).

With emphasis on Nrf2 deficient mice, the genetic absence of Nrf2 does not appear to affect murine development. However, they do develop complex disease manifestations which are similar to that of human autoimmune SLE. This is typified by female dominance, multi-organ inflammation, presence of nuclear auto-antigens and immune complex deposition in blood vessels and glomerular nephritis (Ma et al., 2006). Nrf2 deficiency results in a lowered basal and compromised induction of antioxidant genes e.g. HO-1 and GCLC as observed in a variety of immune cells such as bone marrow cells (BM) (Merchant et al., 2011) myeloid-derived suppressor cells (MDSCs) (Satoh et al., 2010), DCs (Williams et al., 2008), macrophages (Kong et al., 2010; Kong et al., 2011), neutrophils (Kong et al., 2011) and T cells (Rangasamy et al., 2005), and other cell types including hepatocytes (Xu et al., 2008), epithelial cells (Reddy et al., 2007b) keratinocytes (Schafer et al., 2010) and mouse embryonic fibroblasts (MEFs) (Thimmulappa et al., 2006a).

Nrf2 has been shown to protect against hyperoxia-induced acute lung injury and susceptibility to bacterial infection (Reddy et al., 2009a; Reddy et al., 2009b), diesel exhaust particle-induced allergic airway inflammation (Li et al., 2008; Li et al., 2010b), cigarette smoke-induced increase in severity of viral flu infection and associated lung inflammation (Yageta et al., 2011), ovalbumin-induced airway hyper responsiveness and pathogenesis of asthma (Rangasamy et al., 2005), cigarette smoke-induced emphysema (Sussan et al., 2009), LPS-induced sepsis-associated morbidity (Thimmulappa et al., 2006a; Thimmulappa et al., 2006b; Kong et al., 2010) LPS-induced neuroinflammation (Innamorato et al., 2008) and the onset of EAE disease (Johnson et al., 2010).

Synthetic triterpenoids including 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO), its imidazolidine derivative CDDO-Im and particularly the methyl ester CDDO-Me (also known as Bardoxolone methyl) have all proven to potently induce the Nrf2 signalling pathway, resulting in the enhancement of Nrf2 antioxidant genes such including NQO1 and HO-1 (Liby et al., 2005; Yates et al., 2007). These triterpenoid

derivatives provide an effective tool for investigating the role of Nrf2 in the maintenance of oxidative stress. Pre-treatment with CDDO-Im attenuates LPS-induced proinflammatory cytokine production and ROS generation in human PBMCs and neutrophils, respectively (Thimmulappa et al., 2007). Furthermore, CDDO-Im reduces cigarette smoke-induced oxidative stress in the lungs of Nrf2^{+/+} but not Nrf2^{-/-} mice, highlighting the Nrf2-dependent mechanism involved in the inhibition of oxidative stress in this model (Sussan et al., 2009). Additionally, CDDO-Me/Bardoloxone treatment reduces the induction of proinflammatory cytokines following *in vivo* LPS challenge in mice, which is associated with improved mortality (Auletta et al., 2010). Furthermore, it has been shown to delay acute GVHD through suppression of donor T cell expansion after bone marrow transplantation (Li et al., 2010a).

Taken together, this suggests in addition to its role in the maintenance of cellular redox, Nrf2 is a key negative regulator of inflammation, conferring protection against the onset of variety of diseases.

1.17 The role of Nrf2 in cellular adaptation to xenobiotic incursion

As stated previously Nrf2 plays a critical role in the detoxification of drugs and protection against xenobiotic stress, though the induction of many phase II enzymes and in upregulation of glutathione synthesis (Ma and He, 2012). Nrf2 has been shown to protect against acetaminophen-induced hepatotoxicity (Enomoto et al., 2001; Goldring et al., 2004), cisplatin-induced nephrotoxicity (Aleksunes et al., 2010) and bleomycin-induced pulmonary fibrosis (Cho et al., 2004; Kikuchi et al., 2010). This reveals a potential role of Nrf2 in the modulation of adverse drug reactions (ADRs). Adverse drug reactions are a significant health problem contributing to 6.5% of hospital admissions and resulting in post market drug attrition (Pirmohamed et al., 2004). The majority of these ADRs (80%) are predictable relating to the pharmacological action of the drug, which are easily managed clinically through drug cessation (Edwards and Aronson, 2000). However, a proportion of ADRs (20%) are unpredictable and are associated with high mortality (Edwards and Aronson, 2000). These idiosyncratic reactions are generally

immune-mediated e.g. Sulfamethoxazole-induced eczema (Castrejon et al., 2010). Understanding the mechanism behind these off target ADRs is further complicated due to inter-individual variation in responses to various drugs ranging from mild to severe reactions (Edwards and Aronson, 2000). An important issue to consider is whether inter-individual variation in responses to various drugs ranging from Nrf2-mediated adaptation to severe ADRs could be correlated with variability in the Nrf2 pathway induction in humans? This requires intense investigation.

1.18 Role of Nrf2 in human immune-mediated diseases

Nrf2 has been implicated in a variety of immune-mediated human diseases, including chronic obstructive pulmonary disease (COPD) (Malhotra et al., 2008; Suzuki et al., 2008), asthma (Fitzpatrick et al., 2011), MS (van Horssen et al., 2010), nephritis (Cordova et al., 2010), *Helicobacter pylori*-induced gastritis (Arisawa et al., 2007) and ulcerative colitis (Arisawa et al., 2008). Furthermore, it has been suggested that Nrf2 dysfunction plays a pivotal role in the immunological decline associated with the aging process (Kim et al., 2007; Suzuki et al., 2008). Immunosenesescence is associated with chronic levels of oxidative stress resulting in immune cell dysfunction, decreased immunosurveillance, and autoimmunity (Ponnappan and Ponnappan, 2011).

1.19 Role of Nrf2 in immune cell function

It appears clear from the aforementioned evidence presented, that Nrf2 plays key roles in cellular physiology and pathology. Its precise role in immune cell function, however, is not well established and requires further investigation. Nrf2 has been shown to regulate innate immune responses in a murine sepsis model (Thimmulappa et al., 2006a). Furthermore, targeted deletion of Keap-1 in murine myeloid cells prevented sepsis morbidity (Kong et al., 2011). Nrf2 has been shown to regulate macrophage-mediated bacterial clearance and proinflammatory cytokine production of IL-6, TNF α in hyperoxia-induced lung injury (Reddy et al., 2009a). Chemokines e.g. CCL11, CXCL10, CXCL2, which are important innate immune cell chemoattractants and mediators of inflammation, are also

downregulated by Nrf2 and GSH, further highlighting the vital role of Nrf2 in the regulation of the innate immune responses (Reddy et al., 2007b).

The role of Nrf2 in DC immune function has not been fully elucidated. A study by the Biswal laboratory group in which they exposed Nrf2 deficient murine DCs to ambient particulate matter (PM), suggested that Nrf2 plays a role in DC maturation through regulation of co-stimulatory receptor expression of CD80, CD86 and MHCII and DC endocytic function (Williams et al., 2008). Treatment with the antioxidant N-acetylcysteine (NAC), which has been shown to replenish GSH levels, resulted in a down modulation of co-stimulatory receptor expression in both resting and PM-activated DCs (Williams et al., 2008). This finding points towards a role of GSH in maintaining DC function. Furthermore, Nrf2 is implicated in the regulation DC cytokine production and DC-mediated antigen-specific Th1/Th2 differentiation in response to oxidative stress (Williams et al., 2008). Although the groundwork has been laid out in the role of Nrf2 in particular aspects of DC function many questions remain unanswered. How does Nrf2 affect murine DC immune function? Is it through GSH regulation? What role does Nrf2 play in DC phagocytic function in relation to the uptake of apoptotic and necrotic cells? Does it influence DC antigen cross-presentation, DC-mediated antigen-specific CD8 T cell activation, proliferation and subsequent effector function? Is Nrf2 implicated in the regulation of DC-mediated peripheral CD8 T cell tolerance?

The role of Nrf2 in T cell immune function is also poorly understood. A study by the same group depicted a similar role of Nrf2 in regulating Th2 differentiation in an ovalbumin (OVA) allergen-driven asthma murine model (Rangasamy et al., 2005). Nrf2 deficient OVA-challenged splenic CD4 T cells exhibited enhanced production of IL-4 and IL-13 in response to CD3 and CD28 restimulation (Rangasamy et al., 2005). Taken together, this illustrates the emerging role of Nrf2 in the maintenance of Th1/Th2 balance in response to oxidative stress. Numerous points need to be addressed in relation to the role of Nrf2 in T cell immune function. Does Nrf2 play a role in T cell development in the thymus and in peripheral lymphoid organs? What role does Nrf2 play in naïve and effector T cell activation proliferative capacity?

Does it affect T cell polarisation in relation to other CD4 subtypes such as the newly identified Th17?

In line with a previously stated issue that needs to be addressed is the role of Nrf2 mediated adaptive responses to xenobiotic stress in humans. Does human inter-individual variation in Nrf2 adaptive responses to xenobiotics underlie another mechanism that gives rise to differential responses to drugs ranging from adaptation to ADRs? In order to begin answering these complex questions a suitable model must first be established to assess inter-individual variation in the the Nrf2 pathway in response to a known Nrf2 probe.

1.20 THESIS AIMS

The main scope of this thesis is based on testing the hypothesis that Nrf2 regulates DC and T cell immune function. We have utilised the Nrf2 deficient mouse as the experimental system for exploring the role of Nrf2 in DC and T cell immune cell function. This thesis proposes to address the aforementioned questions in relation to DC antigen acquisition capacity, presentation and T cell stimulatory capabilities. Furthermore it aims to investigate the role of Nrf2 in T cell development, activation and T cell polarisation with a concluding focus on the Th17 subtype. The final chapter of the thesis is grounded on the hypothesis that inter-individual variation in Nrf2 activity in response to a known Nrf2 inducer exists in humans. This chapter proposes to investigate variation in the Nrf2 pathway and downstream events in human immune cells derived from healthy subjects in response to the chemical Nrf2 inducer, CDDO-Me.

CHAPTER TWO

METHODS

2.1 Materials and Reagents

Nunclon cell culture flasks and multi-well plates were purchased from Nalge-Nunc International [c/o VWR International, Lutterworth, UK]. Microbiological petri dishes were purchased from sterilin [Newport, UK]. All chemicals and reagents were of analytical grade and purchased from Sigma-Aldrich [Dorset, UK], unless otherwise stated. Foetal Bovine Serum (FBS) was purchased from Gibco [Paisley, UK]. LymphoprepTM was purchased from Axis-Shield [Kimbolton, UK].

2.2 Antibodies

For murine T cell stimulation, anti-mouse CD3 and CD28 antibodies were purchased from eBioscience [Hatfield, UK]. The intracellular fluorescent dye, carboxyfluorescein succinimidyl ester (CFSE) and fluorescein (FITC)-dextran [40,000 MW] were both purchased from Invitrogen [Paisley, UK]. For flow cytometric analyses of cell surface molecules, FITC-conjugated anti-mouse CD40 (Clone 3/23), FITC-conjugated anti-mouse CD86 (GL1), PE-conjugated anti-mouse I-A/I-E (M5/114.15.2), FITC-conjugated anti-mouse CD62L (MEL-14), FITC-conjugated anti-mouse CD69 (H1.2F3), FITC-conjugated anti-mouse CD44 (IM7), APC-conjugated anti-human CD3 (UCHT1) were all purchased from BD Biosciences [Oxford, UK]. FITC-conjugated anti-mouse CD4 (RM4-5), PE-conjugated anti-mouse CD4 (RM4-5), TC-conjugated anti-mouse CD8 (5H10), PE-Cy[®]5.5 conjugated anti-mouse CD11c (N418), FITC-conjugated Annexin V, Alexafluor[®]488-conjugated anti-mouse CD25 (PC61 5.3) were all purchased from Invitrogen [Paisley, UK]. FITC-conjugated anti-human CD11b (ICRF44) was purchased from eBioscience [Hatfield, UK]. For intracellular fluorescence activated cell staining (FACS) staining, FITC-conjugated anti-mouse IFN γ (XMG1.2) and PE-conjugated anti-mouse IL-17A (eBio17B7) were purchased from BD Biosciences [Oxford, UK] and eBioscience [Hatfield, UK], respectively. For western blotting anti-human Nuclear factor-erythroid 2 (NF-E2)–related factor 2 (Nrf2) antibody (EP1808Y- Carboxyterminal end) and anti-mouse beta actin antibody (AC-15) were purchased from Abcam [Cambridge, UK]. The HRP-conjugated anti-mouse and anti-rabbit antibodies were purchased from Sigma-Aldrich [Dorset, UK].

2.3 Peptides and cytokines

NP68 (366ASNENMDAM374) and NP34 (ASNENMETM) nonameric peptides derived from the virus A/NT/60/68 nucleoprotein used to stimulate F5 specific CD8 T cells, were synthesised by Peptide Protein Research Ltd [Fareham, UK]. Recombinant human IL-2, IL-1 β , TGF β , IL-6 and recombinant mouse GM-CSF were all purchased from PeproTech [London, UK]. Mouse recombinant IL-23 was purchased from R&D systems [Abingdon, UK].

2.4 Mice

Black C57BL/6J wild type (Nrf2^{+/+}) and Nrf2 deficient (Nrf2^{-/-}) mice were obtained from Riken BRC (Ibaraki, Japan) (Itoh et al., 1997). Nrf2^{-/-} colonies were maintained as heterozygous breeders and homozygous offspring were routinely genotyped via allele specific polymerase chain reaction (PCR) assays. Mice (C57BL/6) homozygous for the H-2Db-restricted TCR- $\alpha\beta$ transgene, F5, which specifically recognizes NP68 and NP34 nonameric peptides, were a kind gift from Dr James Matthews [Cardiff, Wales]. All animals were housed under controlled conditions at the Biomedical Services Unit, University of Liverpool. All experiments were undertaken in accordance with criteria outlined in licenses granted under the Animals (Scientific Procedures) Act of 1986 (PPL 40/2937 and PPL 40/2544) and approved by the Animal Ethics Committee of the University of Liverpool. Animals were euthanized under schedule 1 carbon dioxide (CO₂) induced asphyxiation procedure and femora, tibiae, thymi, spleens and lymph nodes (axillary, brachial, inguinal and mesenteric) were removed.

2.5 Complete medium

Complete medium (CM) was composed of the following; Roswell Park Memorial Institute-1640 (RPMI-1640) medium, 10 % FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μ g/ml penicillin- 10,000 U/ml streptomycin, 50 μ M 2-mercaptoethanol (2-ME) and 2 mM HEPES solution.

2.6 Cell lines

The human leukemic Jurkat T cell line, E6.1 was purchased from American Type Culture Collection (ATCC). These cells were utilised for the dendritic cell phagocytosis assay (see **section 2.15.3**). Cells were cultured in CM at a density of 0.5^6 cells / ml in nunclon cell culture flasks and media was changed every two days until required for functional assays.

2.7 Generation of Bone Marrow Derived Dendritic cells (BMDCs)

Bone marrow (BM) cells were flushed from femora and tibiae of Nrf2^{+/+} and Nrf2^{-/-} mice. Cells were washed with Hank's Balanced Salt Solution (HBSS), resuspended in CM and viable cells were counted using a haemocytometer and trypan blue exclusion. Bone marrow cells at a density of 3×10^6 /plate were then cultured in microbiological petri dishes containing 10 ml of CM supplemented with 20ng/ml GM-CSF. Cultures were kept in a fully humidified incubator at 37 °C under 5 % CO₂. CM was changed on day 3 and differentiated immature dendritic cells (iDCs) were used between days 6 or 7 for functional assays.

2.8 Glutathione assay

Dendritic cell total glutathione levels (reduced glutathione (GSH) + oxidised glutathione (GSSG)) were determined using a microtitre plate assay. In brief, DCs were washed with HBSS and resuspended in 400 µl 10 mM HCl. Appropriate aliquots were taken to enable the determination of total protein content (Bradford assay). To the remaining samples, sulphosalicylic acid was added at a final concentration of 1.3% (w/v), and protein precipitation was facilitated by incubating on ice for 10 min. Protein was then pelleted by centrifugation at 14000 g for 5 min. The supernatant was removed and used to determine total glutathione content spectrophotometrically at 412 nm using 5,5'-dithio-bis(2-nitrobenzoic acid) (DNTB), β-nicotinamide adenine dinucleotide phosphate (NADPH) and GSH reductase, as described by (Vandeputte et al., 1994) The results were compared to GSH standards (0-50 nMol/ml), and were normalised to protein content using Bradford assay.

2.9 Harvesting of thymocytes, splenocytes and lymph node cells

Isolated thymi, spleens and lymph nodes (inguinal, brachial axillary and mesenteric) excised from Nrf2^{+/+} and Nrf2^{-/-} mice and spleens excised from F5 TCR transgenic mice, were homogenised using Jencons Uniform Grade B-24 Tissue Homogeniser [Jencons Scientific Ltd., Leighton Buzzard, UK]. Dispersed tissue was then filtered through 40 µm gauge Nylon Cell Strainers [BD Falcon, Oxford, UK] and washed with HBSS in two consecutive 5 minute washing/centrifugation cycles. Thymocytes and lymph nodes were centrifuged at 400 x g and 900 x g, respectively. Splenocytes were centrifuged at 300 x g and a red cell lysis step was incorporated between the washing cycles to concentrate nucleated white blood cells. Herein, the cell pellet of the first wash was treated with red blood cell (RBC) lysis buffer (8.3 g/L ammonium chloride in 0.01 M Tris-HCl buffer) for 6 min at RT (25 °C) and quenched using ice cold CM. These cells were then filtered before the second washing step. All cell pellets were re-suspended in CM after the final wash. Viable cells were counted by trypan blue exclusion using a haemocytometer and microscope (Leica DME).

2.10 Immunomagnetic separation of CD4⁺ T cells

Purified CD4 positive Nrf2^{+/+} and Nrf2^{-/-} T cells were isolated using MACS LS⁺ immunomagnetic separation columns [Miltenyi Biotec, Bisley, UK]. Splenocytes were washed and resuspended at a density of 1×10^6 cells per 90 µl of ice cold MACS buffer (HBSS without phenol red supplemented with 2 mM EDTA and 0.5 % bovine serum albumin (BSA)). Cells were magnetically labelled using anti-CD4 conjugated magnetic microbeads [MACS Microbeads, Miltenyi Biotec, Bisley, UK] 10 µl per 1×10^6 cells, and refrigerated for 15 min. Cells were washed with MACS buffer (2ml per 1×10^6 cells), centrifuged at 300 g for 10 min and resuspended in 500 µl MACS buffer. MACS LS⁺ Separation Columns were attached to MidiMACS magnets and set up on a MidiMACS stand. The columns were washed three times with 3 ml of MACS buffer. The magnetically labelled cell suspensions were pipetted onto the column. Unlabelled cells run through and were collected as the negative fraction whereas labelled cells were retained within the column. A thorough elution of the negative fraction was facilitated by washing the columns three times with 3

ml of MACS buffer. The columns were then removed from the magnet and flushed with 5ml of buffer using a column plunger. The magnetically retained CD4⁺ cells were eluted as the positive fraction with a percentage purity of approximately 90 % (data not shown).

2.11 Immunomagnetic separation of CD11c⁺ DCs (For ELISA only)

Purified CD11c positive Nrf2^{+/+} and Nrf2^{-/-} BMDCs were isolated using MACS LS⁺ immunomagnetic separation columns [MACS Microbeads, Miltenyi Biotec, Bisley, UK]. Cells were washed and resuspended at a density of 1×10^6 cells per 80 μ l of ice cold MACS Buffer. Cells were magnetically labelled using anti-CD11c conjugated magnetic microbeads 20 μ l per 1×10^6 cells, and refrigerated for 15 min. Cell separation proceeded as above. This procedure yielded positive cell populations of approximately 90 % purity (data not shown).

2.12 Murine Nrf2^{+/+} and Nrf2^{-/-} effector T cell set up

For T cell stimulation anti-mouse CD3 (2 μ g/ml) and CD28 (2 μ g/ml) mAbs were immobilized by wet-coating onto a 24 well microtitre plate. Processed Nrf2^{+/+} and Nrf2^{-/-} CD4 T cells were seeded at a density of 0.5×10^6 per well in CM for 72 h at 37 °C under 5 % CO₂. Cells were then washed twice with HBSS and re-cultured in CM supplemented with 100 U/ml IL-2 at a density of 1×10^6 cells/ml for 3-4 days. Functional assays were carried out on day 6-7. The initial T cell experiments were conducted using non-purified stimulated Nrf2^{+/+} and Nrf2^{-/-} splenocytes. Purified CD4⁺ T cells were used for experiments requiring Th17 cell polarisation. The Th17 polarising conditions were created by supplementing the CM with 50 ng/ml IL-1 β , 10 ng/ml IL-23, 5 ng/ml TGF β and 25 ng/ml of IL-6 (Mangan et al., 2006; Veldhoen et al., 2009).

2.13 Dendritic cell-mediated F5 antigen-specific T cell proliferation assay

Immature DCs derived from Nrf2^{+/+} and Nrf2^{-/-} mice were pulsed with increasing concentrations of antigenic peptide NP68 or partial agonist NP34 for 2 h at 37 °C. Harvested F5 transgenic murine splenic T cells were added to a 96 well U-bottom microtitre plate (1×10^5 cells per well). Murine F5 CD8 T cells specifically recognise

the NP68 antigenic peptide or NP34 presented by the C57BL/6J BMDCs on the mouse MHC Class I molecule H-2D_b. The pre-pulsed iDCs were washed with HBSS and co-cultured (5,000 BMDC per well) with the F5 CD8 T cells in quadruplicate at 37 °C for 18 h. Murine T cell mitogen Concanavalin-A (CON-A, 4 µg/ml) was used as a positive control. Tritiated-thymidine [Moravek Biochemicals, Brea, CA, U.S.A] (0.5 µCi per well) was added to the wells for the last 16 h of culture. Cells were harvested onto glass-fibre filter mats using a plate harvester [TomTec Mach III, Hamden, CT, USA] and counted in the scintillation counter [Microbeta Trilux, Perkin Elmer, Waltham, MA, USA] after 72 h of incubation.

2.14 Cross presentation assay

Immature DCs derived from Nrf2^{+/+} and Nrf2^{-/-} mice were added to 24 well plates at a density of 2×10^6 cells per well. Cells were allowed to adhere for 2 h at 37 °C and 1 µg/ml of LPS was added during the final 30 min of adherence to induce DC maturation. Cells were washed twice with warm CM in preparation for addition of necrotic C57BL/6J splenocytes. Harvested murine C57BL/6J splenocytes were pulsed with or without NP68 (200µM) for 2 h at 37 °C. Unbound NP68 peptide was removed by extensive washing with HBSS. Necrosis was induced by snap freezing in liquid nitrogen. Necrotic cells were then co-cultured with adherent DCs at a ratio of 2:1 and incubated at 37 °C under 5 % CO₂ for 24h. Cells were then washed and co-cultured in quadruplicate with splenic F5 CD8 T cells (1×10^5 cells/well) at increasing DC:T cell ratios (1:1000 – 1:2) in 96-well U-bottom microtitre plates. Splenic T cell proliferation was measured as above.

2.15 Fluorescence activated cell staining

All protocols in this section were performed using flow cytometric analyses. All cells were acquired on a BD FACSCanto II flow cytometer [BD Biosciences, Oxford, UK] and the post acquisition analysis of flow cytometry data (FCS) files was done using Cyflogic version 1.2.1 software (CyFlo Ltd).

2.15.1 Cell surface receptor expression

For DC phenotyping, cells were scraped from petri dishes and washed in HBSS, counted and dispensed into FACS tubes at a density of 1×10^6 cells/tube. Cells were washed with stain buffer (sheath fluid [BD Biosciences, Oxford, UK] supplemented with 2 % FBS and 2 mM EDTA). Cells were then resuspended in 50 μ l stain buffer and incubated in the dark at 4 °C for 10 min. Appropriate amounts of CD40^{FITC}, CD86^{FITC} and I-A/I-E^{PE} in combination with CD11c^{PE-Cy®5.5} were dispensed to each tube in 50 μ l stain buffer and tubes incubated for 30 min in the dark at 4 °C. After incubation, cells were washed twice with 4ml sheath fluid and resuspended in 700 μ l sheath fluid prior to analyses via flow cytometry.

For splenic, thymic and lymph node T cell phenotyping, cells were processed and dispensed into FACS tubes at a density of 1×10^6 cells/tube. Cell surface staining was performed as above using combination of CD4^{PE}, CD8^{TC} and CD25^{FITC} antibodies. Splenic T cell activation status was also assessed using CD62L^{FITC}, CD69^{FITC} and CD44^{FITC} antibodies in combination with CD4^{PE} and CD8^{TC}.

2.15.2 Dendritic cell endocytosis assay

Endocytic ability of Nrf2^{+/+} and Nrf2^{-/-} iDCs was determined using Dextran^{FITC} as previously described (Sallusto et al., 1995). Harvested iDCs were resuspended at a density of 1×10^6 cells in 100 μ l CM and incubated with 0.5 μ g/ml Dextran^{FITC} (40,000 MW) for 60 min at 37 °C or 4 °C (as negative control). Cells were washed twice in stain buffer, cell surface staining for CD11c^{PE-Cy®5.5} was performed as above and analysed via flow cytometry.

2.15.3 Dendritic cell phagocytosis assays

Nrf2^{+/+} and Nrf2^{-/-} iDCs were scraped from petri dishes, washed in HBSS, counted and plated out into 24 well plates at a density of 2×10^6 cells per well. Cells were allowed to adhere for 2 h at 37 °C. Cells were washed twice with warm CM in preparation for addition of apoptotic or necrotic cells.

To assess phagocytosis of apoptotic cells, processed thymocytes derived from C57BL/6J mice were incubated with 1 μ M dexamethasone at 37 °C under 5 % CO₂ for 18 h. Dexamethasone induces apoptosis in murine thymocytes (Cifone et al., 1999). The proportion of apoptotic thymocytes was determined by staining with FITC Annexin V. Over 90% of thymocytes stained positive for Annexin V (as illustrated in **Figure 3.4**). Apoptotic thymocytes were labelled with CFSE (2 μ M) for 20 min at 37 °C in the dark. Cells were washed with media to remove non-incorporated dye and then co-cultured with adherent iDCs at a ratio of 2:1 for 2 h at 37 °C.

To assess the phagocytosis of necrotic cells, Jurkat T cells were fluorescently labelled with 0.5 μ M CFSE for 30 min at 37 °C in the dark and necrosis was induced via snap freeze in liquid nitrogen. More than 90 % of cells became necrotic as determined by trypan blue exclusion (data not shown). Necrotic cells were co-cultured with DCs as above.

Cells were then harvested, washed with stain buffer and stained with CD11c^{PE-Cy5.5} prior to analysis by flow cytometry.

2.15.4 Annexin V assay

The extent of apoptosis on dexamethasone-treated thymocytes was determined using the FITC Annexin V Apoptosis Detection Kit [BD Biosciences, Oxford, UK] according to the manufacturer's instructions. One of the early events in apoptosis is the inversion of the cell's phospholipid bilayer resulting in the exposure of phosphatidylserine molecules. These molecules are readily detected by Annexin V antibody (Fadok et al., 1992). Briefly, dexamethasone-treated C57BL/6J thymocytes (1×10^6 cells per FACS tube) were washed with 1X binding buffer, centrifuged and resuspended in 100 μ l of 1X binding buffer. Cells were gently vortexed and incubated with Annexin V^{FITC} antibody for 15 min at RT in the dark. Cells were then washed with 1X binding buffer, centrifuged, resuspended in binding buffer and analysed via flow cytometry.

2.15.5 Interferon- γ intracellular staining for DC mediated re-stimulation of F5 CD8 T cells

Immature DCs derived from Nrf2^{+/+} and Nrf2^{-/-} mice were scraped from petri dishes, washed in HBSS and counted. Immature DCs were pulsed with or without NP68 (1×10^{-7} M) for 2 h at 37 °C. Cells were then extensively washed to remove unbound NP68 and plated out in a 6 well plate (300,000 DCs per well). Cells were allowed to adhere for 2 h at 37 °C. Processed F5 CD8 T cells were co-cultured with the adherent iDCs at a density of 7×10^6 cells per well for 72h at 37 °C under 5 % CO₂. These F5 CD8 T cells were then re-stimulated in 24 well plate with mature Nrf2^{+/+} DCs (treated with 1 μ g/ml LPS overnight) which were either pulsed or unpulsed with NP68 (1×10^{-7} M). This co-culture was at a density of 1×10^6 F5 CD8 T cells and 50,000 Nrf2^{+/+} mDCs per well. Cells were co-cultured for 6 h in total. Golgi plug inhibitor Brefeldin A (contained within Cytofix/Cytoperm kit as stated below) was added 1 hour after start of culture to allow intracellular cytokine accumulation. Cells were then stained with CD8^{TC} antibody as per 2.15.1 method. Intracellular cytokine staining for IFN γ was achieved using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit with BD GolgiPlug™ [BD Biosciences, Oxford, UK] according to manufacturer's instructions. After cell surface staining, cells were washed with stain buffer and fixed & permeabilised in one step using 1x BD Cytofix/Cytoperm solution for 20 min at 4 °C in the dark. Cells were washed twice with 1x BD Perm/Wash™ buffer and incubated with IFN γ ^{FITC} plus 1x BD Perm/Wash™ buffer for 30 min at 4 °C in the dark, followed by two final washes with 1x BD Perm/Wash™ buffer and resuspended in 2-4% paraformaldehyde (PFA). Flow cytometric analysis was performed the following day

2.15.6 Effector T cell IFN γ intracellular staining

Day 6 Nrf2^{+/+} and Nrf2^{-/-} effector T cells were re-stimulated in 24 well plate at a density of a density of 1×10^6 T cells per well with plate bound anti-mouse CD3 (2 μ g/ml) and CD28 (2 μ g/ml) antibodies for 6 h. Unstimulated cells were used as a background control. Golgi plug inhibitor Brefeldin A (contained within Cytofix/Cytoperm kit as stated above) was added 1 hour after start of culture to

allow intracellular cytokine accumulation. Cells were then stained with CD8^{TC} antibody as per 2.15.1 method. IFN γ intracellular staining was performed as above.

2.15.7 Effector T cell IL-17A/IFN γ intracellular staining

Day 6 Nrf2^{+/+} and Nrf2^{-/-} effector CD4⁺ T cells were re-stimulated in a 24 well plate at a density of 1×10^6 T cells per well either under non-polarizing or Th17 polarizing conditions with 50 ng/ml PMA, 1 μ g/ml ionomycin and plate bound anti-mouse CD3 (2 μ g/ml). Cells that were left without stimulation were used as background control. Golgi plug inhibitor Brefeldin A (contained within Cytofix/Cytoperm kit as stated above) was added 1 hour after start of culture to allow intracellular cytokine accumulation. Cells were then stained with CD4^{R-PE} antibody as per 2.15.1 method. Interferon- γ intracellular staining was performed as above with addition of IL-17A^{PerCp-Cy5.5} antibody.

2.15.8 Measurement of reactive oxygen species

Processed Nrf2^{+/+} and Nrf2^{-/-} splenocytes were washed with HBSS and resuspended in serum-free RPMI 1640. Subsequently, cells (5×10^5 cells/ tube) were treated with or without H₂O₂ for 10 minutes, washed and resuspended in HBSS without phenol red. The ROS indicator dihydroethidium (DHE) (10 μ M) was then added to the appropriate tubes and incubated at 37°C for 10 minutes. Dihydroethidium is a colourless substance that can freely diffuse across the cell membrane. Upon oxidation with intracellular superoxide radicals specifically, DHE forms into an oxidised red fluorescent product which is easily detected by flow cytometric analysis (Burnaugh et al., 2007). Immediately after DHE incubation, cells were analysed on a flow cytometer.

2.16 Antibody stimulated T cell proliferation assay

Tissue culture 96 well plate was treated with increasing equivalent concentrations of anti-mouse CD3 and CD28 antibodies (0-1 μ g/ml) in 50 μ l HBSS for 2 h at 37 °C to allow for antibody binding. Non-plate bound antibody was washed off with HBSS in preparation for T cell treatment. Processed Nrf2^{+/+} and Nrf2^{-/-} T cells were dispensed onto the plate bound antibody treated plate (50,000 T cells per well) in

CM and incubated for 72 h at 37 °C under 5 % CO₂. CON-A (4 µg/ml) was used as a positive control. Tritiated-thymidine (0.5 µCi per well) was added to the wells for the last 16h of culture. Cells were harvested onto glass fibre filter mats using and counted in the scintillation counter after 72h of incubation.

2.17 Dendritic cell Enzyme-linked immunosorbent assay (ELISA) sample preparation

Day 6 Purified CD11c⁺ Nrf2^{+/+} and Nrf2^{-/-} DCs were plated in 2 ml CM supplemented with 3 % GMCSF at 0.8 x 10⁶ cells/ml on a 24 well plate. 1 µg/ml LPS were added to appropriate wells to induce DC maturation. Cells were incubated at 37 °C under 5 % CO₂ for 24 h. Cells were then spun down at 900 x g for 5 min and sample supernatants were stored at -80 °C in preparation for enzyme-linked immunosorbent assay (ELISA).

2.18 Murine T cell ELISA sample preparation

Day 6 effector Nrf2^{+/+} and Nrf2^{-/-} DCs T cells were re-stimulated with plate bound anti-mouse 2 µg/ml CD3 and 1 µg/ml CD28 antibodies in a 24 well plate (1 x 10⁶ cells/ml) in CM supplemented with 100 U/ml IL-2. Cells were incubated at 37 °C under 5 % CO₂ for 24 h. Cells were pelleted at 700 x g for 5 min and sample supernatants were stored at -80 °C in preparation for ELISA.

2.19 Measurement of cytokine production by ELISA.

The levels of murine IL-12p70, IL-2, IFNγ, IL-13 and TNFα were measured using mouse IL-12p70, IL-2, IFNγ, IL-13 and TNFα Quantikine ELISA kits, respectively [R&D systems, Abingdon, UK]. The levels of murine IL-17A, IL-5 and IL-22 were measured using mouse IL-17A, IL-5 and IL-22 ELISA Ready-SET-Go kits, respectively. All experiments were performed through strict adherence to manufacturer's instructions. To briefly explain the procedure for the Ready-SET-Go sandwich immunoassays, a 96-well ELISA plate was initially coated with the relevant capture antibody in coating buffer (provided in kit) and incubated at 4 °C overnight. The following day, the plate was washed three time with ELISA wash buffer (0.5 % TWEEN in 1 x PBS) and tapped dried to remove the remaining wash buffer from the

wells. This was followed by two hour incubation with blocking buffer (provided in kit) at RT. The plate was then washed as described earlier. Relevant standards were added by serial dilution to the designated wells and finally 50 μ L of supernatant was added to the appropriate wells and incubated for 2 h at RT. The plate was then washed thoroughly as before and incubated with detection antibody for 1 hour. This was followed by careful washing cycles and incubation with the enzyme concentrate; avidin-HRP in the dark for 20 min. The plate was again washed six times and incubated in the dark for 20 min with 100 μ L of TMB substrate. This reaction was quenched by the addition of 100 μ L of 1M H_2SO_4 . The OD (Optical Density) was then measured using a spectrophotometer [Varioskan, Thermo scientific Hudson, NH, USA] at 450 nm. Based on the raw OD₄₅₀ data obtained, a standard curve was plotted and the concentrations of each cytokine were then calculated. The above method is relatively similar for the Quantikine ELISAs, however, kits were supplied with ELISA plates that were pre-coated with the relevant capture antibodies and all reagents required for the immunoassays were included in the kits.

2.20 Isolation of human Peripheral blood mononuclear cells (PBMCs)

Blood was taken from 15 healthy donors (age range 20-35; 7 male and 8 female) after signed informed consent. This was approved by the Local Research Ethics Committee. Human PBMCs were isolated from 50ml of heparinised venous whole blood using LymphoprepTM (density of 1.077 g/ml, 9.1 % (w/v) sodium diatrizoate and 5.7 % (w/v) polysaccharide) density gradient centrifugation. Cells of lower density such as PBMCs form a distinct band above the LymphoprepTM layer. In contrast, RBCs and granulocytes which have a higher density collect at the bottom of the LymphoprepTM layer. In brief, the heparinised blood was gently layered onto an equal volume of LymphoprepTM. The blood was then centrifuged at 700 g without brakes for 25min at RT. The PBMC layer above the LymphoprepTM was gently aspirated and washed three times with HBSS. Cells were then resuspended in CM and viable cells were counted by trypan blue exclusion using a haemocytometer and microscope.

2.21 Human T cell activation

Isolated human PBMCs were activated with the human T cell mitogen phytohaemagglutinin (PHA, 2.5 µg/ml) in CM supplemented with 360 U/ml IL-2 at a density of 2×10^6 cells per ml in a culture flask. Human T cells were allowed to expand for 72h at 37 °C under 5 % CO₂. Cells were then washed twice with HBSS and returned to culture with CM supplemented with 100 U/ml IL-2 for a further 48 h. T cell purity was determined by human CD3^{APC} cell surface staining and flow cytometric analysis. This procedure yielded CD3 positive cell populations of 85% and above (represented in **Figure 5.1A**). Human T cells were used on day 5 for functional assays.

2.22 Detergent lysis of human cells

Day 5 stimulated human T cells and fresh human neutrophils were harvested and resuspended in serum-free RPMI 1640. Cells (5×10^6) were treated with increasing concentrations of CDDO-Me (0-100 nM) for 2 h at 37 °C. Cells were then centrifuged and lysed in 500 µL 1x radio-immunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1.0 % IGEPAL® CA-630, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris, pH 8.0) plus protease inhibitors (10 µg/ml Aprotinin, 10 µg/ml Leupeptin and 10 µg/ml Pepstatin A) for 30 min on ice. Cells were then centrifuged at 16000 x g for 10 min at 4 °C. Supernatants were removed and stored at -80 °C for future use.

2.23 Nuclear extraction of human T cells

Day 5 stimulated human T cells were harvested, washed twice with HBSS and resuspended in serum-free RPMI 1640. 8×10^6 T cells were dispensed into eppendorfs and treated with increasing concentrations of CDDO-Me (0-100nM) to induce Nrf2 activation for 2 h at 37°C. The proteasome inhibitor MG132 (100 nM) which prevents Nrf2 proteasomal degradation resulting in enhanced nuclear Nrf2 accumulation (McMahon et al., 2003) was used as a positive control for Nrf2 induction. Nuclear Lysates were achieved using NE-PER nuclear and cytoplasmic extraction kit [Fischer Scientific, Loughborough, UK] via strict adherence to

manufacturer's instructions. Briefly, 8×10^6 CDDO-Me treated T cells were centrifuged, supernatant was removed and pellets resuspended in 100 μ L Cytoplasmic extraction reagent I (CER I) plus protease inhibitors. Cells were left on ice for 10 min after which 5.5 μ L CER II was added to each eppendorf, vortexed and left on ice for a further 1 min. Samples were then spun at 16,000 g at 4°C for 5min and the supernatant containing cytoplasmic fractions were removed and stored at -80°C for future use. The remaining pellets were resuspended in 50 μ L Nuclear extraction reagent (NER) and left on ice for 45min with intermittent vortexing every 15 min. Cells were then spun at 16,000 g at 4°C for 10min and the supernatant containing nuclear fractions was carefully removed and stored at -80°C for future use.

2.24 Protein content determination via Bradford assay

Whole cell lysates, nuclear extract from cell samples and HCl treated cells were generated as required for each experimental protocol. The total protein content of samples was determined using Protein Assay Dye Reagent [Bio-rad; Hemel Hempstead, UK] according to the manufacturer's instructions. This assay relies on the binding of Coomassie Brilliant Blue G-250 dye to basic and aromatic amino acids, resulting in a change in colour of the dye (red to blue) (Bradford and Ward, 1976). This change in the dye colour is proportional to the amount of bound dye and can be measured in the absorbance at 570 nm with a MRX plate reader [Dynex; Lincoln, UK]. Total protein content of samples was calculated from the absorbance values of a 0.25-5 μ g BSA standard curve prepared for each assay.

2.25 Western blotting: Human Nrf2 protein expression

Whole cell lysates and nuclear extracts (20 μ g as determined by the Bradford assay) were denatured via the addition of 5 μ L loading buffer (70 % (v/v) NuPAGE sample loading buffer, 30 % (v/v) NuPAGE reducing agent) and incubated at 80 °C for 5 min. Denatured samples were loaded onto pre cast 15 lane NuPAGE® 4-12% Bis Tris gels [Life Technologies, Paisley, UK] alongside PrecisionPlus protein Kaleidoscope standards. Samples were resolved by electrophoresis in a X Cell Surelock mini-cell using a 3-(N-morpholino) propanesulphonic acid (MOPS) running buffer (50 mM

MOPS, 50 mM Tris base, 3.5 mM sodium dodecyl sulphate, 1 mM EDTA, 0.25 % (v/v) NuPAGE antioxidant), at 90 V for 10 min, followed by 80 min at 170 V. Resolved proteins were transferred onto nitrocellulose membrane [GE Healthcare Life Sciences, Buckinghamshire, UK] at 200mA for 90min with ice. Subsequently, nitrocellulose membranes were stained with Ponceau S solution to allow visibility of resolved proteins. Membranes were then cut along the 50 kDa marker and blocked overnight in a covered container at 4 °C in blocking buffer consisting of Tris-buffered saline (TBS)-Tween (TBST) solution ((TBS; 0.15 M NaCl, 25 mM Tris base, 3 mM KCl, pH 7.0) with 0.1 % (v/v) Tween 20) containing 10 % non-fat milk powder. For Nrf2 expression, membranes were treated with primary anti-human Nrf2 antibody at a concentration of 1:400 in TBST containing 2 % non-fat milk powder for 3 h. For actin expression, membranes were treated with primary anti-mouse beta-actin antibody at concentration of 1:40,000 in TBST containing 2 % non-fat milk powder for 30 min. Membranes were then washed 4 times with TBST for 15 min intervals. Membranes were then treated with anti-rabbit (for Nrf2) and anti-mouse (for actin) secondary antibodies for 1 hour at 1:10,000 in TBST containing 2 % non-fat milk powder and washed as before. Finally, immunoblots were visualised using Western Lightning® Plus-ECL, Enhanced Chemiluminescence Substrate reagents [Perkin Elmer, Buckinghamshire, UK] and exposed to Hyperfilm ECL [GE healthcare Life Sciences, Buckinghamshire UK] under darkroom conditions, using a Kodak BioMax MS intensifying screen. Blots were developed using Kodak developer and fixer solutions.

Films were scanned using a GS-710 calibrated imaging densitometer [Bio-rad; Hemel Hempstead, UK], immunoreactive band volumes were quantified using TotalLab 100 software [Total lab Ltd; Garth heads, Newcastle upon Tyne, UK], in accordance with the manufacturer's instructions, and normalised to β -actin.

2.26 Sample preparation for RNA extraction

Day 5 human stimulated T cells were harvested, washed twice with HBSS and resuspended in CM supplemented with 100 U/ml IL-2. Cells were plated out at 2×10^6 cells per well in a 24 well plate and treated with 50 nM CDDO-Me over a 0-24

hour time course. Subsequently, cells were washed twice with HBSS and pellets were lysed in 600 µl RLT buffer [Qiagen, UK] containing 1 % 2-ME and stored at -80°C for RNA extraction.

2.27 Ribonucleic acid extraction

Total Ribonucleic acid (RNA) was isolated from CDDO-Me stimulated human T cell lysates using the RNeasy Mini kit [Qiagen, UK] according to manufacturer's instructions. All surfaces and equipment were rendered RNase-free, by wiping with RNasezap, prior to the isolation of RNA. Deoxyribonucleic acid (DNA) was digested during the RNA purification process using DNase derived from the RNase-free DNase set [Qiagen, UK]. Briefly, lysates were spun at maximum speed, transferred to RNase free microcentrifuge tubes and mixed with 1 volume of 70 % ethanol. The mixture was then transferred onto the spin columns and centrifuged for 15 seconds at 8000 x g. High salt concentration and ethanol containing buffers were sequentially added to the columns followed by centrifugation. Finally, RNA was eluted from the membrane via centrifugation using 30 µl RNase free water. The concentration and purity of each RNA sample was assessed spectrophotometrically using a NanoDrop ND-1000 [Thermo Scientific, UK]. The purity of RNA in each sample was determined via reference to the 260/280 nm ratio, as protein is detected at 280 nm. RNA samples with a 260/280nm ratio of below 2 were rejected as impure. RNA was stored at -80 °C until required.

2.28 Complementary DNA synthesis

Ribonucleic acid was reverse-transcribed to complementary DNA (cDNA) using the ImPromptII reverse transcription system [Promega, Southampton, UK] according to manufacturer's instructions. Reactions (20 µL) containing 1 µg RNA, 0.5 µg random primer and nuclease free water were heated at 70°C for 5 min and rapidly cooled on ice. 20 µL of reverse transcriptase reaction stock solution (2.5 % 5X reaction buffer, 8 mM MgCl₂, 0.5 mM deoxyribonucleotide triphosphate (dNTP), 1 % reverse transcriptase, nuclease free water) was added to each sample immediately after and heated at 25 °C for 5 min, followed by 42 °C for 60 min and 70 °C for 15 min.

Samples were then rapidly cooled on ice and 80 µL nuclease free water added to each tube. Samples were stored at -80 °C until required.

2.29 Real-time PCR

Complementary DNA (1.5 µL, approximately 0.1 µg) was combined with 10 µL of 2X SYBR green jumpstart TaqMan ready mix (includes the intercalating fluorescent dye SYBR green that binds to double stranded DNA) [Sigma, UK], 2 µL of forward and reverse primers for human NAD(P)H:quinine oxidoreductase 1 (*NQO1*) (forward sequence 5'-TCCAGTTCCCCTGCAGTGGT-3' and reverse sequence 5'-CCGTGGATCCCTTGCAGAGAGT-3') or the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (forward sequence 5'-CAAGTCATCCATGACAACCTTG-3' and reverse sequence 5'-GGGCCDTCCACAGTCTTCTG-3') and 5.5 µL DNase/RNase free water in a clear 96-well PCR microplate plate [Greiner Bio-One Ltd, Stonehouse, UK]. Plates were sealed with Absolute QPCR seals, using an adhesive seal applicator, and briefly centrifuged at 900 x g to remove air bubbles. Gene expression was analysed by quantitative real-time PCR on an ABI PRISM 7000 Sequence Detection System, in accordance with the manufacturer's instructions. Levels of *NQO1* gene expression were calculated via reference to their cross threshold (CT) values and normalised to *GAPDH*.

2.30 Statistical analysis

Where appropriate, experiments were performed in triplicate, and all experiments were replicated on separate occasions. Depending on the results set, data are expressed as either mean \pm standard deviation of the mean (SD) or mean \pm standard error of the mean (SEM). One-way analysis of variance (ANOVA), with Tukey's post-test applied, the unpaired Student's *t* test or the Mann-Whitney U test were used to assess the significance of any differences in the data compared to appropriate controls. A two-sided *P* value of ≤ 0.05 was considered to be statistically significant.

CHAPTER THREE

THE ROLE OF NRF2 IN DENDRITIC CELL IMMUNE FUNCTION

3.1 INTRODUCTION

Maintenance of the DC intracellular redox status is imperative for the execution of appropriate DC immune function (Chan et al., 2006). Cellular redox disequilibrium involving oxidative stress is known to induce DC maturation and alter DC cytokine production, further influencing the nature of the adaptive immune response elicited (Kantengwa et al., 2003; Ohtani et al., 2005; Chan et al., 2006). The powerful antioxidant GSH plays a crucial role in the nullification of oxidative stress. It is therefore implicated in the regulation of DC redox homeostasis (Kim et al., 2007). Glutathione biosynthesis is governed by the transcriptional activity of Nrf2 (Copple, 2012). The redox sensitive transcription factor Nrf2 is responsible for the induction of an array of cytoprotective and anti-oxidant defence genes e.g. GCLC and NQO1 in response to oxidative and electrophilic insult, thus plays an important role in regulating cellular redox homeostasis (Copple, 2012). Within DCs, Nrf2 regulates co-stimulatory receptor expression and DC-mediated Th1/Th2 polarisation (Williams et al., 2008; Rangasamy et al., 2010). However, its precise role in DC immune function has not been fully elucidated and requires intense investigation. It is unknown whether Nrf2 plays a role in regulating the capacity of DC antigen acquisition or whether the effect of Nrf2 on DC phenotype and function is attributable to their basal GSH content. Furthermore, the role of Nrf2 in DC-mediated antigen-specific CD8 T cell activation and subsequent effector function has yet to be established. The hypothesis of the work described in this chapter is that Nrf2 regulates DC immune function. Using the Nrf2 deficient mouse model as a means by which to test this hypothesis, the following phenotypic and functional parameters were investigated: role of Nrf2 and DC GSH content on DC co-stimulatory receptor expression, antigen acquisition and DC-mediated CD8 T cell stimulatory capacity. Furthermore, the influence of Nrf2 on the ability of DCs to cross-present cell associated antigens to naïve CD8 T cells and its role in activated DC cytokine secretion were also examined.

3.2 RESULTS

3.2.1 Loss of Nrf2 results in lowered basal GSH content in immature DCs

The redox sensitive transcription factor Nrf2 is a key player in the regulation of GSH biosynthesis in response to oxidative insult (Reddy et al., 2007b). It is recognised that loss of Nrf2 results in the inhibition of the basal and inducible expression of genes necessary for GSH synthesis including the GCL rate limiting enzyme subunits GCLm and GCLc (Reddy et al., 2007b; Williams et al., 2008). Although it has been established that Nrf2 deficient mice exhibit diminished GSH levels in a variety of cell types including hepatocytes (Toyama et al., 2011), mouse embryonic fibroblasts (MEFs) (Harvey et al., 2009), lung epithelial cells (Reddy et al., 2007a), thymocytes (Morito et al., 2003) and macrophages (Kong et al., 2010), it has not been assessed in DCs. Accordingly, we hypothesised that loss of Nrf2 in DCs would result in lowered GSH levels and thus examined total reduced GSH content in Nrf2^{+/+} and Nrf2^{-/-} iDCs. Results revealed that Nrf2^{-/-} bone marrow-derived iDCs exhibited a significant decrease in GSH levels in relation to their wild type counterpart as illustrated in **Figure 3.1** (Nrf2^{+/+} 36.6 ± 11.5 nmol/mg versus Nrf2^{-/-} 24.4 ± 7.8 nmol/mg, $p < 0.05$), thus highlighting the pivotal role of Nrf2 in the maintenance of GSH content and potentially redox homeostasis within DCs.

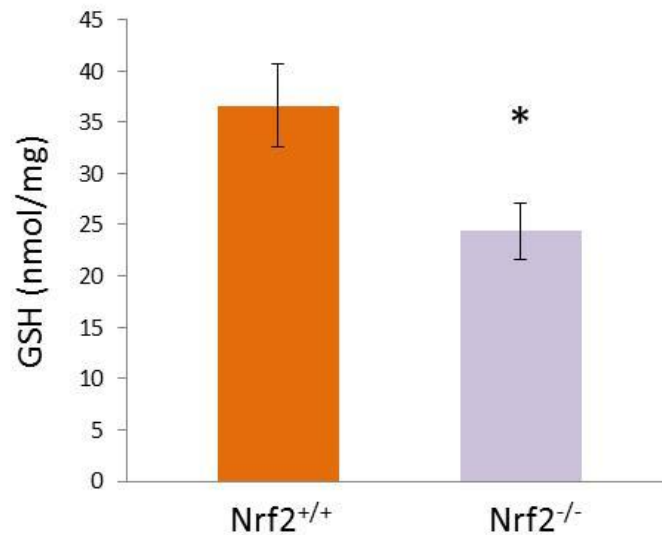


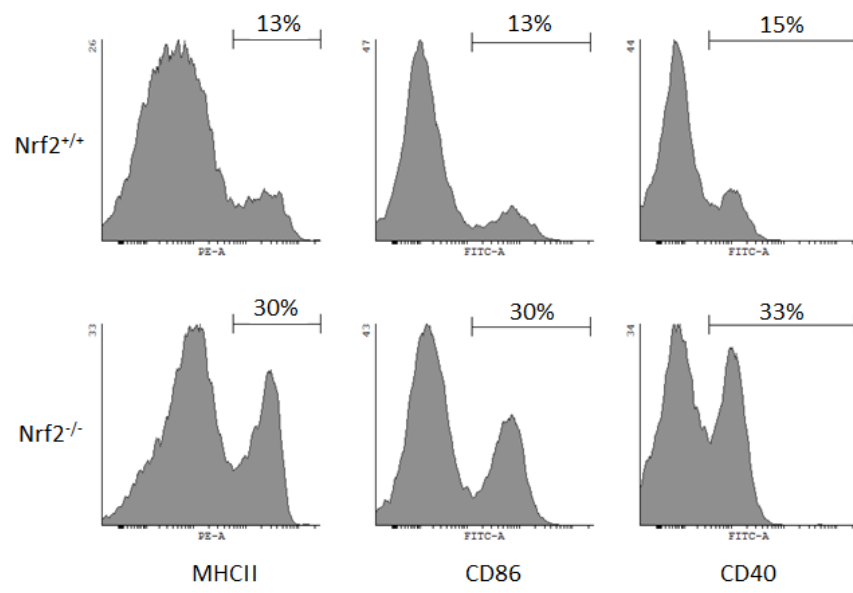
Figure 3.1 Loss of Nrf2 results in attenuation of basal GSH content in immature DCs. Basal GSH levels were measured in resting bone marrow-derived dendritic cells generated from Nrf2^{+/+} and Nrf2^{-/-} mice. Total amount of GSH was normalised to total protein content. Error bars represent S.E.M. Statistical significance was tested by unpaired Student's *t* test. Data is derived from 3 independent experiments (*, *p* < 0.05).

3.2.2 Enhanced co-stimulatory expression in Nrf2 deficient DCs is not a direct consequence of reduced GSH levels

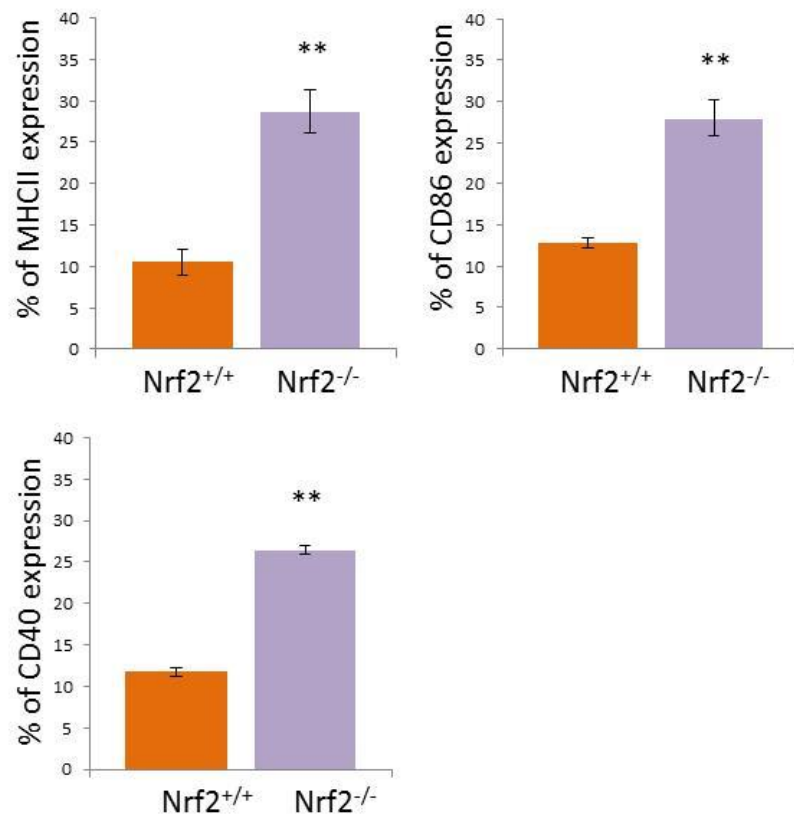
It is known that DC intracellular redox influences DC maturation events through the regulation of DC co-stimulatory molecule expression (Kantengwa et al., 2003; Matsue et al., 2003). Previous studies have illustrated a role of intracellular GSH levels in governing DC co-stimulatory expression (Verhasselt et al., 1999; Mizuashi et al., 2005; Kim et al., 2007; Williams et al., 2008). Furthermore, it has been established that Nrf2 deficient iDCs exhibit enhanced co-stimulatory receptor expression of CD86 and MHCII (Williams et al., 2008). We have illustrated that loss of Nrf2 results in reduced intracellular GSH levels. However, it is unknown whether the changes in co-stimulatory receptor expression observed in the Nrf2 deficient iDCs are simply attributable to the observed lowered GSH content or rather due to an Nrf2 deficient cell intrinsic effect. Initially, we attempted to reaffirm the findings presented in the aforementioned study concluding that loss of Nrf2 mediates enhanced co-stimulatory receptor expression in iDCs (Williams et al., 2008). Immature DCs are composed of subpopulations that express low or high levels of co-stimulatory molecules (**Figure 3.2Ai**). We focussed our flow cytometric analysis on Nrf2^{+/+} and Nrf2^{-/-} iDCs expressing high levels of co-stimulatory molecules (**Figure**

3.2Ai, highlighted underneath markers) as changes in these subpopulations would highly influence the DC maturation status and their subsequent immune function. Results confirmed that Nrf2^{-/-} iDCs exhibited a near 3 fold increase in MHCII (Nrf2^{+/+} 10.5 ± 1.6% versus Nrf2^{-/-} 30.5 ± 2.1%, *p* = 0.0003) and more than 2 fold increase in CD86 (Nrf2^{+/+} 12.5 ± 0.6% versus Nrf2^{-/-} 29.8 ± 1.3%, *p* < 0.0001) expression in comparison to the wild type control as quantified in **Figure 3.2Aii**. In contrast to the previous report, we also observed a significant increase in Nrf2^{-/-} iDC CD40 expression (Nrf2^{+/+} 11.8 ± 0.5% versus Nrf2^{-/-} 26.5 ± 0.5%, *p* < 0.0001). Our results therefore confirm that loss of Nrf2 augments co-stimulatory expression in iDCs. Next we investigated the impact of altered GSH levels on iDC co-stimulatory receptor function. We hypothesised that lowering GSH levels in Nrf2^{+/+} iDCs would recapitulate the enhanced maturation phenotype exhibited in the Nrf2 iDCs. To address this, Nrf2^{+/+} iDCs were treated with buthionine sulfoximine (BSO) and intracellular GSH levels were evaluated. Buthionine sulfoximine is a potent inhibitor of the GCLc enzyme (Griffith and Meister, 1979) and has previously been shown to pharmacologically deplete GSH levels in human DCs (Kamide et al., 2011). Results illustrated that BSO significantly reduced GSH intracellular levels in Nrf2^{+/+} iDCs when compared to the untreated control (**Figure 3.2B**, Nrf2^{+/+} 20.4 ± 2.1 nmol/mg versus BSO 24hr 5.1 ± 1.6 nmol/mg, *p* < 0.05). Further examination of MHCII, CD86 and CD40 expression in BSO-treated and untreated Nrf2^{+/+} iDCs revealed no detectable changes in co-stimulatory receptor expression between both groups as depicted as overlay histograms in **Figure 3.2C**. Taken together, our findings suggest that the enhanced maturation phenotype exhibited in the Nrf2 deficient iDCs is not a direct consequence of reduced GSH content.

Ai



Aii



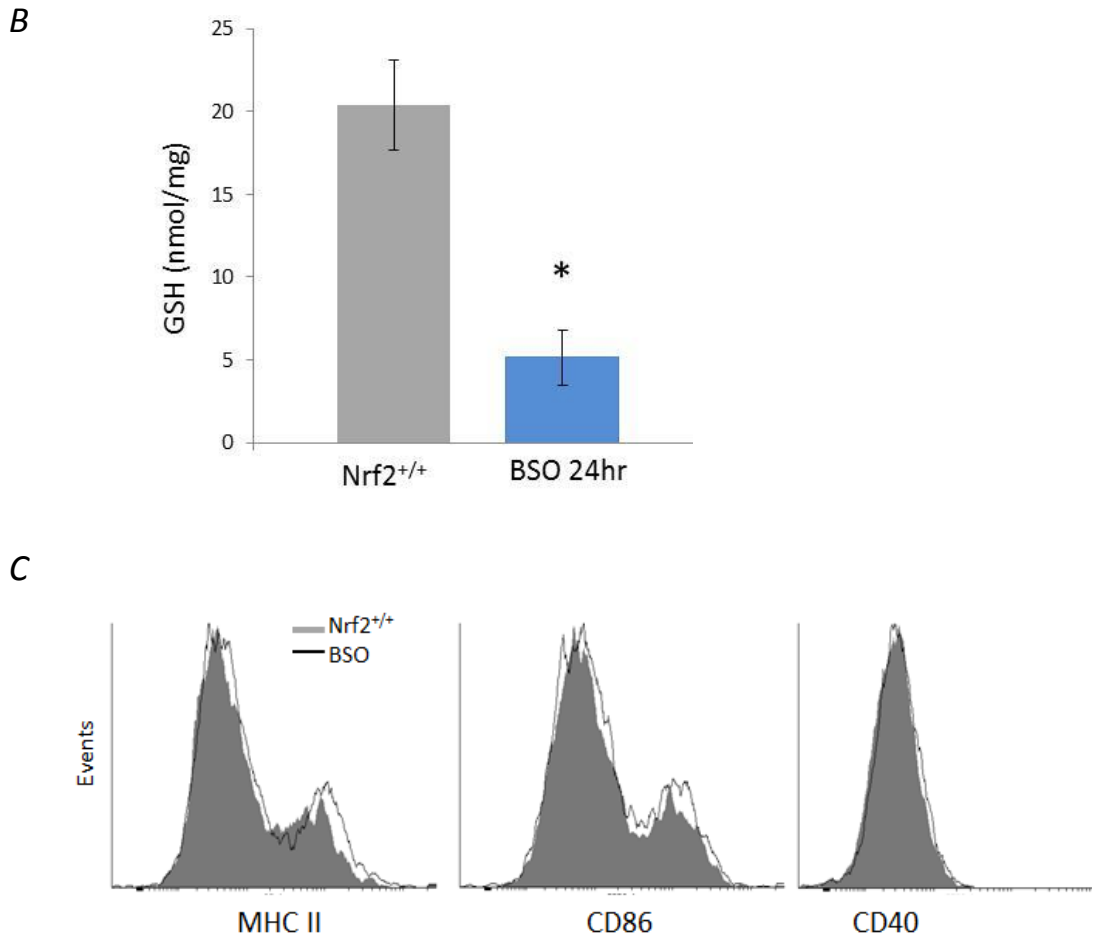


Figure 3.2 Loss of Nrf2 results in a GSH-independent increase in co-stimulatory receptor expression in iDCs. Immature Nrf2^{+/+} and Nrf2^{-/-} iDCs were stained CD11c^{PE-Cy5.5} in conjunction with MHC II^{PE}, CD86^{FITC} and CD40^{FITC} and analysed by flow cytometry. Cell were gated on CD11c⁺ iDCs and percentage of co-stimulatory receptors expression was determined within this population as represented in (Ai) and quantified in (Aii). GSH levels were measured in; **B**. Nrf2^{+/+} iDCs untreated or treated with BSO (100 μ M) for 24h. Error bars S.E.M. **C**. Immature Nrf2^{+/+} DCs untreated or treated with BSO (100 μ M) for 24h were stained CD11c^{PE-Cy5.5} in conjunction with MHC II^{PE}, CD86^{FITC} and CD40^{FITC} and analysed by flow cytometry as above. Histogram overlays show fluorescence intensity of respective co-stimulatory receptors. Statistical significance was tested by unpaired Student's *t* test (*, $p < 0.05$; **, $p < 0.01$). Data are derived from 4 independent experiments.

3.2.3 Loss of Nrf2 impairs endocytic capacity of iDCs independently of lowered GSH levels

The fundamental role of iDCs is to continuously sample their environment for potential infectious pathogens. Thus they are highly efficient at acquiring antigens from the periphery (Banchereau et al., 2000). Immature DCs capture antigens through a variety of endocytic and phagocytic mechanisms including mannose receptor-mediated endocytosis of sugars and glucan carbohydrate structures (Geijtenbeek and Gringhuis, 2009). It is recognised that upon maturation, DCs lose their antigen acquisition capabilities whilst developing into highly immunogenic APCs (Garrett et al., 2000). As we have shown that Nrf2^{-/-} iDCs exhibited an enhanced maturation phenotype associated with increased co-stimulatory receptor expression, we hypothesised that this would be accompanied by a down regulation in endocytic capacity. To examine the latter, Nrf2^{+/+} and Nrf2^{-/-} iDCs were treated with fluorescently conjugated Dextran^{FITC} and analysed by flow cytometry to assess mannose receptor-mediated endocytosis, a routinely utilised method for examining endocytosis (Sallusto et al., 1995). Results demonstrated that Nrf2^{-/-} iDCs had a diminished capacity to endocytose dextran in comparison to their wild type counterpart (**Figure 3.3A**, Nrf2^{+/+} 48.0 ± 8.6% versus Nrf2^{-/-} 84.7 ± 5.4%, $p < 0.01$). Similarly to our previous result, we sought to decipher whether this impairment in endocytosis was a consequence of lowered GSH levels, thus we compared dextran uptake between Nrf2^{+/+} and BSO-treated Nrf2^{+/+} iDCs. Our results revealed no differences in endocytic capacity between the two treatment groups (**Figure 3.3B**). Taken together, this demonstrates that loss of Nrf2 in iDCs results in impairment in antigen acquisition capacity through endocytosis which is not a direct consequence of their lowered cellular GSH content.

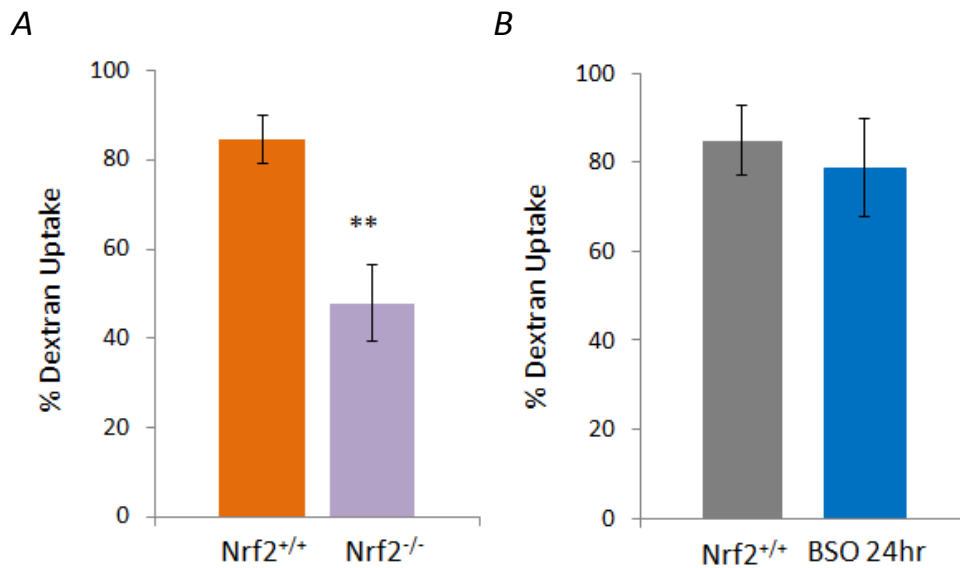


Figure 3.3 Loss of Nrf2 impairs endocytic capacity of iDCs which is not a direct consequence of lowered GSH levels. Nrf2^{+/+} and Nrf2^{-/-} iDCs (A) or Nrf2^{+/+} DCs untreated or treated with BSO (100 μ M) for 24h (B) were incubated with Dextran^{FITC} for 60 min at 37°C. Cells were subsequently stained with CD11c^{PE-Cy5.5} and Dextran^{FITC} uptake by CD11c⁺ gated iDCs was assessed by flow cytometry. Data are presented as average percentage uptake \pm SD. Statistical significance was tested by unpaired Student's *t* test (**, *p* < 0.01). Data are derived from 4 independent experiments.

3.2.4 Loss of Nrf2 results in impaired capacity of iDCs to phagocytose dying cells independently of lowered GSH levels

Immature DCs are also capable of phagocytosing dying cells which have undergone apoptosis as a consequence of normal cellular homeostasis or necrosis due to infection (Bratton and Henson, 2008; Sancho et al., 2009). The ability to ingest apoptotic and necrotic cells by phagocytosis ultimately leads to the induction of DC mediated-CD8 T cell peripheral tolerance or CD8 T cell immunity, respectively (Kurts et al., 1997; Kurts et al., 1998; Sauter et al., 2000; Sancho et al., 2009; Nopora et al., 2012). We reasoned that loss of Nrf2 in iDCs would also impair their ability to phagocytose dying (apoptotic and necrotic) cells. Initially, we assessed the ability of Nrf2^{+/+} and Nrf2^{-/-} iDCs to uptake fluorescently labelled apoptotic thymocytes. This was achieved by treating isolated C57BL/6J thymocytes with dexamethasone overnight to induce apoptosis (Cifone et al., 1999). One of the early events involved in apoptosis is the inversion of the phospholipid bilayer thus exposing

phosphatidylserine molecules. These molecules are readily detected by Annexin V antibody (Fadok et al., 1992). The proportion of apoptotic cells were thus determined by staining with Annexin-V as shown in **Figure 3.4**.

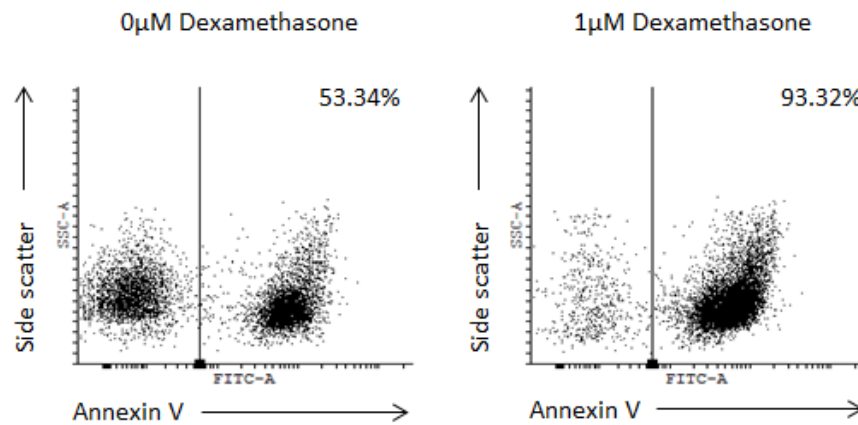


Figure 3.4 Dexamethasone induces apoptosis in murine thymocytes. Isolated C57BL/6J thymocytes were treated with or without 1μM dexamethasone for 24 h. Cells were subsequently stained with Annexin V^{FITC} and analysed by flow cytometry. Percentages of Annexin V⁺ thymocytes are indicated in the right hand panels. Apoptotic thymocytes were required for dendritic cell phagocytosis assay. Data are representative of 3 independent experiments

Apoptotic cells were subsequently fluorescently labelled with CFSE, co-cultured with Nrf2^{+/+} and Nrf2^{-/-} iDCs at 4°C or 37°C and analysed by flow cytometry. Results were converted into fold increase in phagocytosis from the 4°C basal control. Results revealed that Nrf2^{-/-} iDCs had a reduced capacity to phagocytose apoptotic cells in relation to their wild type counterpart (**Figure 3.5Ai**, Nrf2^{+/+} 4.7 ± 1.0 versus Nrf2^{-/-} 2.8 ± 0.4, $p = 0.02$). To test whether GSH content contributed to this diminished phagocytic function, we examined uptake of apoptotic cells in Nrf2^{+/+} and BSO-treated Nrf2^{+/+} iDCs. Results showed no differences in phagocytosis between both treatment groups (**Figure 3.5Aii**). Next we examined Nrf2^{+/+} and Nrf2^{-/-} iDCs capacity to phagocytose necrotic cells. This was achieved by labelling Jurkat cells with CFSE and inducing necrosis via snap freeze in liquid nitrogen. Necrotic cells were subsequently co-cultured with the Nrf2^{+/+} and Nrf2^{-/-} iDCs as before and analysed by flow cytometry. Results highlighted that Nrf2^{-/-} iDCs exhibited a decreased capacity to phagocytose necrotic cells (**Figure 3.5Bi**, Nrf2^{+/+} 4.8 ± 1.8 versus Nrf2^{-/-} 1.7 ± 0.7, $p < 0.01$). We further confirmed that no differences in

phagocytosis of necrotic cells were observed between Nrf2^{+/+} and BSO-treated Nrf2^{+/+} iDCs as illustrated in **Figure 3.5Bii**. Taken together, our results demonstrate that loss of Nrf2 in iDCs results in an enhanced maturation phenotype which is associated with a defect in capacity to phagocytose dying cells. Importantly, our results emphasise that the latter is not a direct consequence of lowered GSH levels.

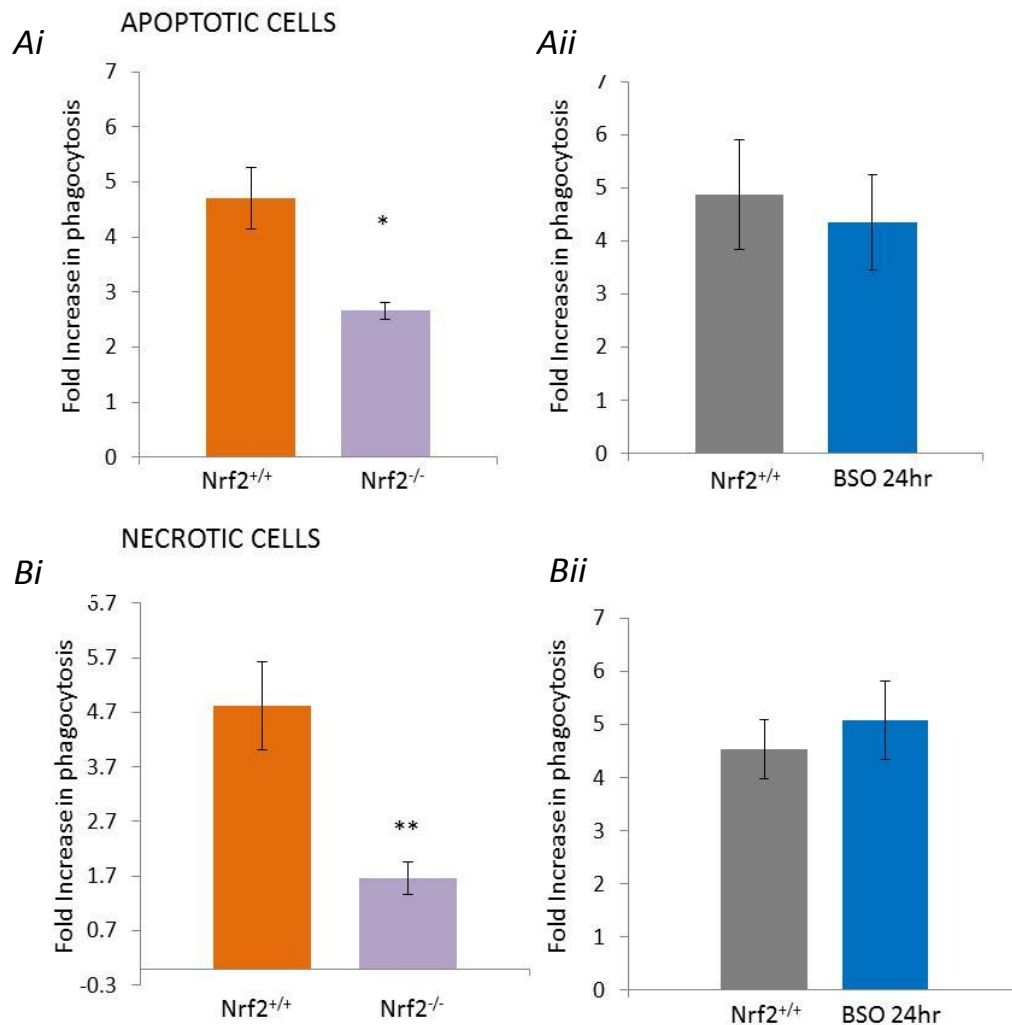


Figure 3.5 Loss of Nrf2 results in impaired capacity of iDCs to phagocytose dying cells which is not a direct consequence of lowered basal GSH levels. Nrf2^{+/+} and Nrf2^{-/-} iDCs (**Fig. 3.5Ai and Bi**) and Nrf2^{+/+} iDCs untreated or treated with BSO (100 μ M) for 24h (**Fig. 3.5Aii and Bii**) were co-cultured with CFSE labelled (A) apoptotic thymocytes or (B) necrotic Jurkat cells at 37°C for 2 hours. DC phagocytic capacity was measured by flow cytometry as an increase in CFSE levels compared to corresponding 4°C baseline control samples. Data is presented as average fold-changes \pm SEM. Statistical significance was tested by the Mann-Whitney U test (*, $p < 0.05$; **, $p < 0.01$). Data are derived from 5 independent experiments.

3.2.5 Enhanced co-stimulatory receptor expression of Nrf2^{-/-} iDCs is associated with enhanced antigen-specific CD8 T cell stimulatory capacity

CD8 T cells provide vital protection against an array of intracellular pathogens including viruses and are also fundamental for the implementation of anti-tumour responses (Barber et al., 2006; Bos et al., 2012; Condotta et al., 2012). Naïve CD8 T cells specifically recognise antigenic peptides bound to MHC class I complexes on DCs via their distinctive T cell receptor (TCR) (Trombetta and Mellman, 2005). Competent CD8 T cell activation only occurs, however, in the presence of additional secondary signals provided through the binding of co-stimulatory molecules present on the surface of the DC and T cell (Bachmann et al., 1997). The low co-stimulatory molecule expression on iDCs renders them inept at inducing competent T cell activation (Banchereau et al., 2000). Given that Nrf2 deficient iDCs express enhanced levels of CD86, CD40 and MHCII, we anticipated that this would equip them to induce an antigen-specific CD8 T cell response. Assessing antigen-specific T cell activation is extremely problematic, however, given the diverse range of T cells expressing distinctive TCRs that specifically recognise an infinite number of antigens. In order to address this issue, we utilized a TCR transgenic mouse model, F5, wherein the majority of CD8 T cells exclusively express a T cell receptor originally derived from a CTL clone, F5 (Mamalaki et al., 1992; Mamalaki et al., 1993). The F5-TCR specifically recognises the MHC I-D^b restricted antigenic peptide NP68, derived from the nucleoprotein of the influenza virus strain A/NT/60/68 (Mamalaki et al., 1992; Mamalaki et al., 1993). The Nrf2^{+/+} and Nrf2^{-/-} iDCs were pre-pulsed with increasing concentrations of NP68 and co-cultured with naïve F5 CD8 T cells. Functional consequences of changes in DC co-stimulatory receptor expression were assessed by the ability of the NP68 bearing DCs to stimulate antigen-specific F5 CD8 T cell proliferation. Results revealed that Nrf2^{+/+} iDCs were unable to induce significant antigen-specific F5 CD8 T cell proliferation until 10nM NP68 which was further increased by over 3-fold at the highest 100nM NP68 concentration (**Figure 3.6Ai**). In contrast, Nrf2^{-/-} iDCs significantly induced CD8 T cell proliferation when pre-pulsed with the lowest 1nM NP68 concentration followed by an approximate 1.5-fold NP68 concentration dependent significant increase in T

cell proliferation (**Figure 3.6Ai**, $p < 0.05$). Nrf2^{-/-} iDCs induced significantly enhanced F5 CD8 T cell proliferation at all given NP68 concentrations in relation to their wild type control (**Figure 3.6Ai**, $p < 0.05$). Similar to the results observed in the previous phenotype and antigen capture experiments, pharmacologically depleting GSH with BSO in Nrf2^{+/+} iDCs did not influence CD8 T cell activation (**Figure 3.6Aii**). Taken together these data demonstrate that the enhanced maturation phenotype of Nrf2^{-/-} iDC is associated with an increased capacity to induce antigen-specific CD8 T cell stimulation and is not a consequence of lowered GSH levels.

Peripheral naive T cells exhibit low affinity for self-peptides and rely on continuous contact with self-peptide:MHC complexes presented by iDCs for their survival (Surh and Sprent, 2008). The iDC phenotype is characterised by low co-stimulatory receptor expression which provides the appropriate signals for T cell survival and maintenance of T cell tolerance (Kurts et al., 1997; Kurts et al., 1998; Steinman et al., 2000). Increases in co-stimulatory receptor expression in iDCs have been shown to induce overt T cells activation which may lead inappropriate autoimmune responses (Hawiger et al., 2001).

As the Nrf2 deficient iDCs exhibit an enhanced maturation phenotype with increased CD40, CD86 and MHCII, we reasoned that this would result in inappropriate T cell activation in response to a self-peptide and breach in self-tolerance. To examine this, Nrf2^{+/+} and Nrf2^{-/-} iDCs were pre-pulsed with increasing concentrations of the altered peptide ligand (APL) NP34. The APL NP34 is a peptide analogue of NP68 in which amino acid residues present within the TCR contact sequence have been modified to lower the affinity of NP34 for the F5 TCR (Williams et al 1996). This APL does not induce F5 CD8 T cell activation and thus simulates to a degree, the interaction between self-peptides with TCRs on naïve CD8 T cells (Williams et al 1996). The results verified that NP34-pulsed Nrf2^{+/+} iDCs failed to induce T cell proliferation at any given NP34 in F5 CD8 T cell proliferation at the 10nM NP34 concentration and above relative to unpulsed iDCs (**Figure 3.6Bi**). Conversely, NP34-pulsed Nrf2^{-/-} iDCs induced a 2-fold increase in F5 CD8 T cell proliferation at the 10nM NP34 concentration and above in comparison to unpulsed iDCs (**Figure 3.6Bi**). There were no differences in F5 CD8 T cell

proliferation observed between BSO-treated and untreated $\text{Nrf2}^{+/+}$ iDCs (**Figure 3.6Bii**). These results demonstrate that the enhanced co-stimulatory receptor expression exhibited by the $\text{Nrf2}^{-/-}$ iDCs is associated with enhanced CD8 T cell proliferation in the context of a self-peptide.

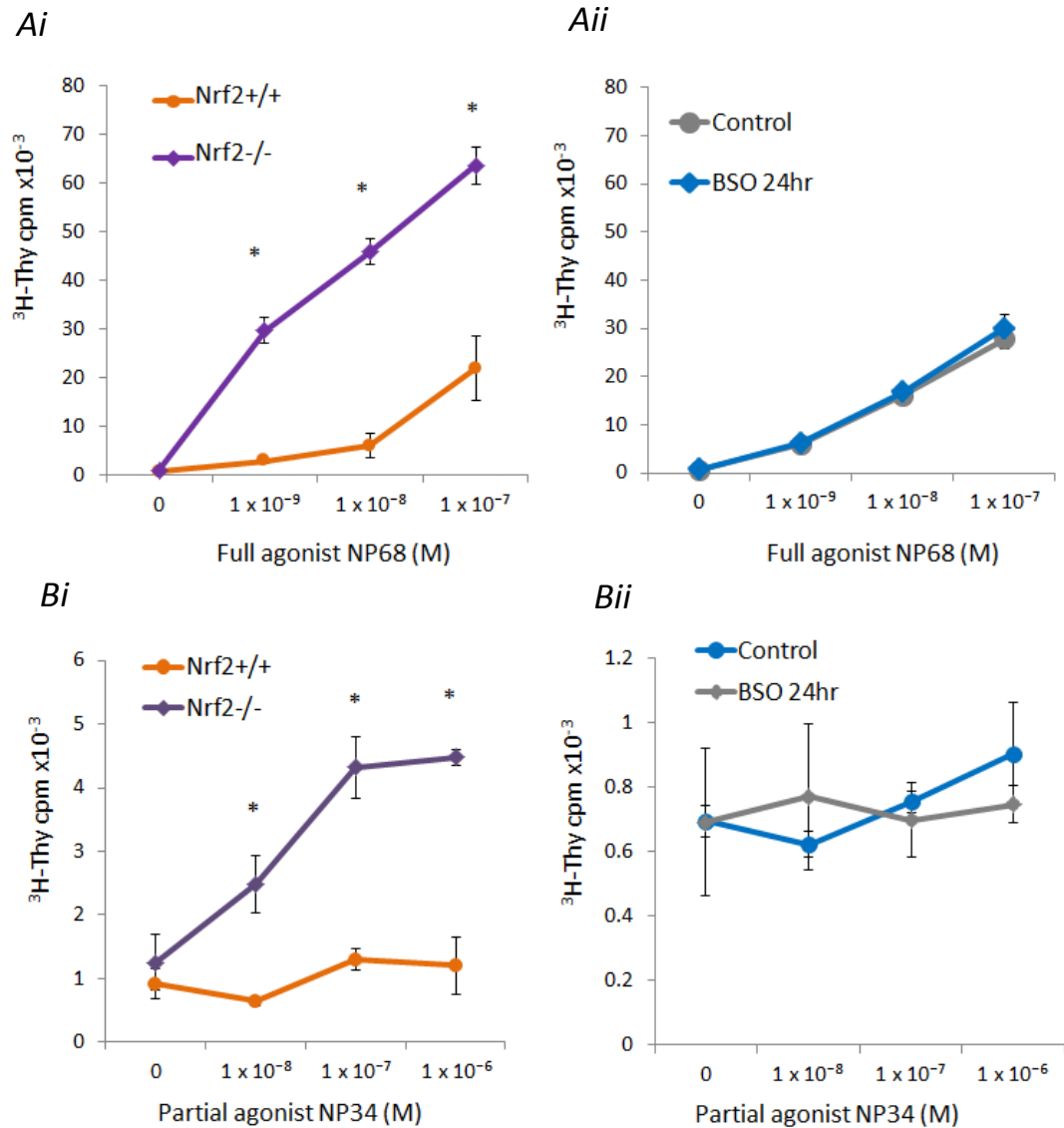
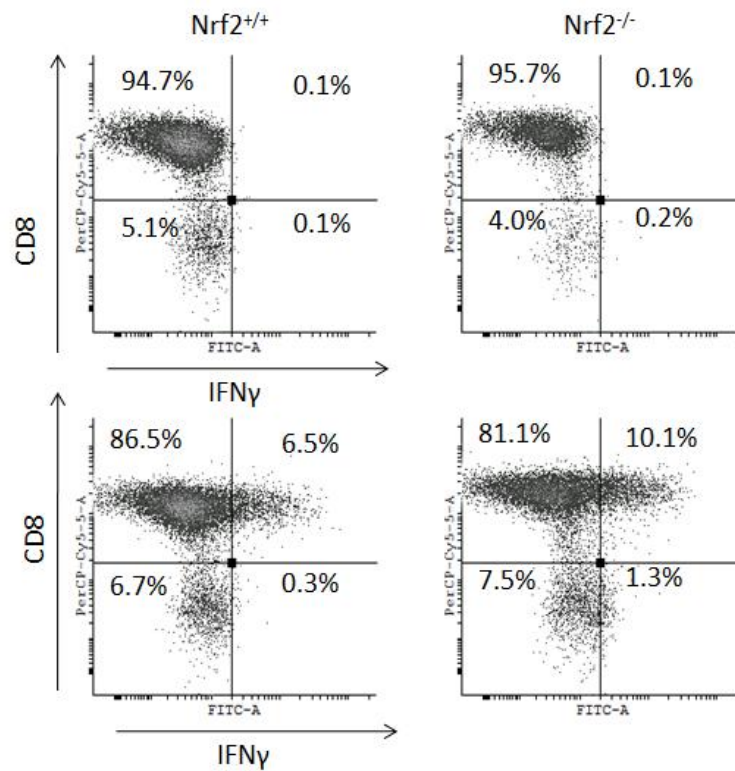


Figure 3.6 Increased co-stimulatory receptor expression by $\text{Nrf2}^{-/-}$ iDCs is associated with enhanced antigen-specific CD8 T cell immunostimulatory capacity. **A.** $\text{Nrf2}^{+/+}$ and $\text{Nrf2}^{-/-}$ iDCs and **B.** Untreated $\text{Nrf2}^{+/+}$ and BSO (100 μM) treated $\text{Nrf2}^{+/+}$ iDCs were pulsed with increasing concentrations of; **(i)** NP68 antigenic peptide or; **(ii)** NP34 altered peptide ligand and were co-cultured with F5 CD8 T cells for 72 hours. ^3H -Thymidine ($^3\text{H-Thy}$) was added for the last 16 hr. Proliferation of T cells was determined by scintillation counting of incorporated ^3H -Thymidine. Data are presented as average [^3H]thymidine scintillation counts \pm S.D. Statistical significance was tested by unpaired Student's t test (*, $p < 0.05$).

3.2.6 Enhanced co-stimulatory receptor expression of Nrf2^{-/-} iDCs is associated with enhanced antigen-specific CD8 T cell effector function

Dendritic cell-activated CD8 T cells undergo several rounds of proliferation followed by functional differentiation into effector CD8⁺ CTLs that characteristically secrete large amounts of the cytokine IFN γ . Co-stimulatory signal transduction is known to enhance the expansion of CD8⁺IFN γ ⁺ producing T cells in response to intracellular bacterial infection (Mittrucker et al., 2001). From our observation that the Nrf2 deficient iDCs exhibit enhanced co-stimulatory receptor expression associated with increased antigen-specific F5 CD8 T cell proliferation, we hypothesised that these Nrf2^{-/-} iDC-activated F5 CD8 T cells would have enhanced effector function in terms of the number of antigen-specific CD8 T cell IFN γ producing effector cells in relation to the Nrf2 wild type iDCs. In order to address this, NP68-prepulsed Nrf2^{+/+} and Nrf2^{-/-} iDCs were co-cultured with naïve F5 CD8 T cells for 72 hours. Antigen-specific CD8 T cells were subsequently restimulated with NP68-pulsed mature Nrf2^{+/+} DCs and intracellular IFN γ production was assessed by flow cytometry. It appeared that Nrf2^{-/-} iDCs induced a small increase in the percentage of antigen-specific CD8⁺IFN γ ⁺ producing T cells in comparison to their wild type control (**Figure 3.7A**, percentage of F5 CD8⁺IFN γ ⁺ T cells, Nrf2^{+/+} 6.5% versus Nrf2^{-/-} 10.1%). However, when comparing the number of CD8⁺IFN γ ⁺ T cells produced from the starting culture of F5 T cells, Nrf2^{-/-} iDCs generated between 1.4- and 2.4-fold enhanced numbers of effectors cells from the naïve F5 CD8 T cells when compared to Nrf2^{+/+} iDCs as illustrated in **Figure 3.7B**. These results highlight the association between increased co-stimulatory receptor expression of Nrf2^{-/-} iDCs with enhanced capacity to induce antigen-specific CD8 T cell functional differentiation into IFN γ producing effector CTLs.

A



B

Animal Pair	Stimulating DC genotype	Starting F5 CD8 T cell numbers	No. of IFN γ producing effectors generated
1	Nrf2 ^{+/+}	7 x 10 ⁶	2.08 x 10 ⁶
	Nrf2 ^{-/-}	7 x 10 ⁶	5.10 x 10 ⁶
2	Nrf2 ^{+/+}	7 x 10 ⁶	3.74 x 10 ⁶
	Nrf2 ^{-/-}	7 x 10 ⁶	6.12 x 10 ⁶
3	Nrf2 ^{+/+}	7 x 10 ⁶	11.40 x 10 ⁶
	Nrf2 ^{-/-}	7 x 10 ⁶	15.76 x 10 ⁶

Figure 3.7 Nrf2 deficient iDCs enhanced maturation phenotype is associated with enhanced DC-mediated antigen-specific CD8 T cell effector function. Naïve F5 CD 8 T cells were co-cultured with Nrf2^{+/+} or Nrf2^{-/-} iDCs pre-pulsed with 10⁻⁷ M NP68 for 72hrs. F5 T cells were then re-stimulated with Nrf2^{+/+} mDC pre-pulsed with or without 10⁻⁷ M NP68 for 6 hrs and assessed for IFN- γ production by intracellular cytokine staining (ICS). **A.** Representative dot plot of IFN- γ ICS for re-stimulated F5 CD8 T cells. Percentages of cells are indicated in the panels. **B.** Collated table of total number of IFN- γ producing effectors generated. Data are derived from 3 independent experiments.

3.2.7 Loss of Nrf2 enables DCs to cross-present cell-associated peptide antigens to CD8 T cells

Generally proteins derived from exogenous material are presented as processed antigenic peptides in conjunction with MHCII molecules to naïve CD4 T cells thus initiating CD4 T cell immune responses (Trombetta and Mellman, 2005). However, it has long been recognised that DCs can uniquely present exogenous cell-associated antigens derived from phagocytosed dying or infected cells in conjunction with MHCI molecules to naïve CD8 T cells through a phenomenon known as “cross-presentation” (den Haan et al., 2000; Belz et al., 2005). Cross-priming mechanisms have been shown to facilitate CD8 anti-viral and anti-tumour immunity (Hoffmann et al., 2000; Nopora et al., 2012). Furthermore, this constitutive process is crucial for maintenance of peripheral CD8 T cell tolerance (Kurts et al., 1997; Kurts et al., 1998). We have previously established that Nrf2 deficient iDCs have a reduced capacity to phagocytose dying cells. We therefore investigated whether this defect in phagocytosis would impact on the ability of Nrf2 deficient DCs to cross-present necrotic cell-associated antigens to CD8 T cells. In order to evaluate the influence of Nrf2 on DC cross-presentation, we adapted the aforementioned F5-TCR experimental system by supplying NP68 pre-pulsed necrotic C57BL/6J splenocytes to LPS-stimulated Nrf2^{+/+} and Nrf2^{-/-} DCs overnight, to enable phagocytosis and the processing of cell-associated NP68 antigen. Subsequently, the DCs were co-cultured with naïve F5 CD8 T cells at varying DC:T cell ratios and the level of antigen-specific CD8 T cell proliferation was used as a direct determinant of the DC cross-presentation capacity. Results revealed that Nrf2^{-/-} DCs were able to cross-present cell-associated NP68 more efficiently than their wild type counterpart as evidenced by the significantly enhanced CD8 T cell proliferation exhibited at increasing DC:T cell ratios from 1:50 to 1:5 (**Figure 3.8**, $p < 0.05$). Intriguingly, Nrf2^{+/+} DCs failed to induce CD8 T cell proliferation at any given DC:T cell density (**Figure 3.8**, stimulation index < 2 at all DC:T cell ratios).

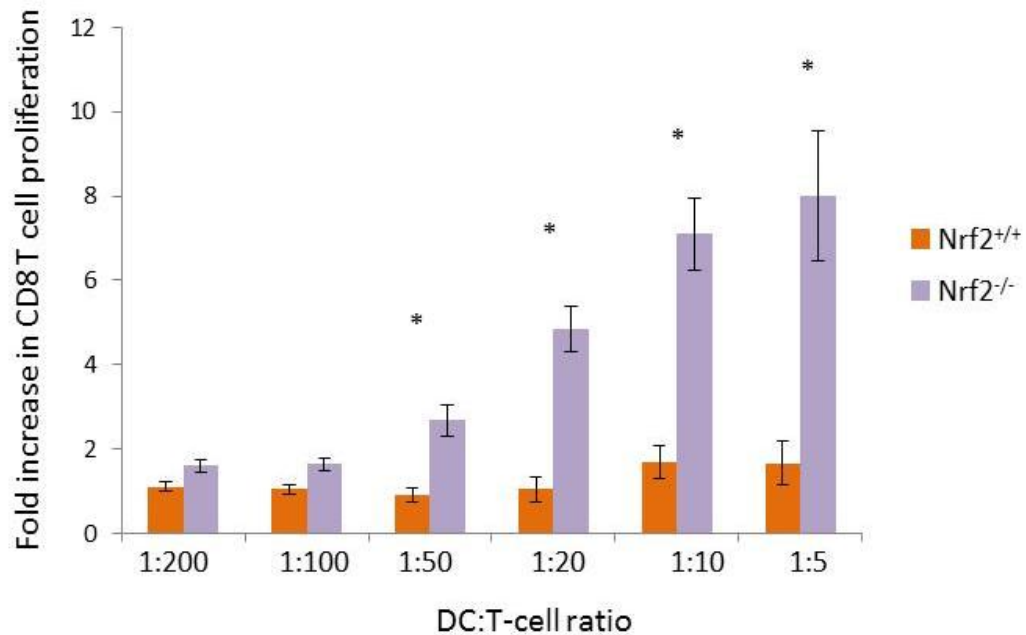


Figure 3.8. Nrf2 deficient iDCs have increased capacity to cross-present cell-associated antigens to naïve CD8 T cells. Necrotic splenocytes preloaded with NP68 were cultured overnight in the presence of LPS with Nrf2^{+/+} and Nrf2^{-/-} iDCs to enable phagocytosis and cross-presentation of cell-associated antigen. Dendritic cells were harvested, washed and co-cultured at various ratios with F5 CD8 T cells for 72 hr. ³H-Thymidine was added for the last 16 hours. Proliferation of T cells was determined by scintillation counting of incorporated ³H-Thymidine by T cells and converted to stimulation indices by dividing total F5 CD8 T cell proliferation activated by 200 μ M NP68 over the control group (0 M NP68). Error bars represent S.E.M. Statistical significance was tested by Mann–Whitney U test (*, $p < 0.05$). Data are derived from 4 independent experiments.

3.2.8 Loss of Nrf2 in activated DCs results in a lowered Th1 cytokine profile

Dendritic cells produce an array of cytokines as a consequence of differential engagement of innate recognition receptors including TLRs (Banchereau et al., 2000). Dendritic cell cytokine production provides a vital tertiary signal to naïve CD4 T cells governing their differentiation into functionally distinct CD4 effector subtypes including the cytotoxic Th1 and allergic Th2 subsets (Kim et al., 2007; Williams et al., 2008). Dendritic cell regulation of Th1/Th2 polarisation is crucial for the normal functioning of the immune system (Maldonado-Lopez and Moser, 2001; Kidd, 2003). Oxidative stress influences DC cytokine production, resulting in a perturbation of the Th1/Th2 balance and permitting a dominant Th2 phenotype as exhibited in a variety of allergic disorders e.g. asthma and eczema (Kim et al., 2007;

Williams et al., 2008; Holgate, 2012). We sought to investigate the impact of loss of Nrf2 on basal and LPS-induced DC Th1 TNF α , IL-12p70 and Th2 IL-2 cytokine secretion. We hypothesised that loss of Nrf2 would enhance Th2 cytokine production within this context. In order to achieve this, we purified day 6 CD11c⁺ Nrf2^{+/+} and Nrf2^{-/-} iDCs by CD11c positive magnetic separation, stimulated the DCs with or without LPS for 48hr and analysed the levels of TNF α , IL-12p70 and IL-2 by ELISA. Results revealed that although the difference is very small, Nrf2^{-/-} basal iDCs secreted significantly lower levels of TNF α in comparison to their wild type control (**Figure 3.9A**, Nrf2^{+/+} 212.6 \pm 7.0 versus Nrf2^{-/-} 191.6 \pm 6.3, p = 0.02). Dendritic cell treatment with LPS resulted in significantly increased TNF α levels for both genotypes (p < 0.002), however similarly to basal findings, LPS-treated Nrf2^{-/-} DCs produced significantly lower TNF α levels in comparison to their wild type control (**Figure 3.9A**, Nrf2^{+/+} 1596.3 \pm 60.6 pg/ml versus Nrf2^{-/-} 936.1 \pm 54.7, p < 0.0001). Basal levels of IL-12 were undetectable for both genotypes (**Figure 3.9B**). Upon LPS stimulation, Nrf2^{-/-} DCs secreted significantly lowered levels of IL-12p70 in relation to their wild type counterpart (**Figure 3.9B**, Nrf2^{+/+} 49.2 \pm 14.7 pg/ml versus Nrf2^{-/-} 19.7 \pm 12.1, p = 0.009). In contrast to the Th1 findings, there were no significant differences LPS-activated DC secretion of IL-2 between Nrf2^{+/+} and Nrf2^{-/-} mice (**Figure 3.9C**, Nrf2^{+/+} 18.7 \pm 2.4 pg/ml versus Nrf2^{-/-} 21.4 \pm 16.6). The Th2 cytokines IL-5 and IL-13 were also measured; however, levels were undetected (IL-15) or below the limit of detection (IL-13) under basal and LPS-induced conditions for both genotypes (data not shown). Taken together, this suggests that under Th1 inducing conditions using the TLR4 agonist LPS, Nrf2 deficient DCs exhibit a lowered Th1 cytokine profile highlighting a potential role of Nrf2 in the regulation of DC-mediated Th1 polarisation.

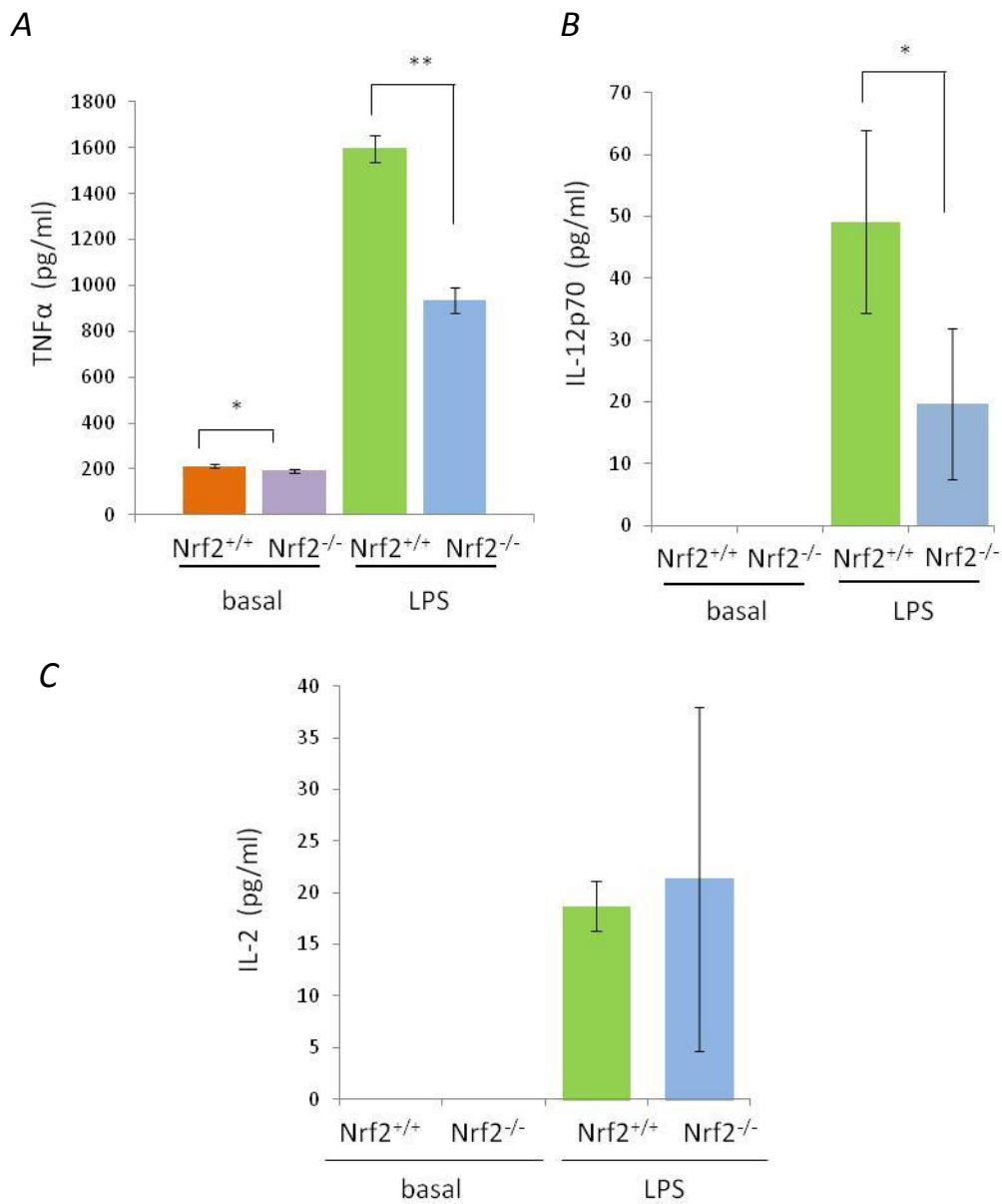


Figure 3.9 Nrf2 deficient DCs exhibit a lowered Th1 cytokine profile

Day 6 CD11c⁺ purified Nrf2^{+/+} and Nrf2^{-/-} iDCs were stimulated with or without LPS for 48hr. Levels of **A.** TNFα, **B.** IL-12p70, **C.** IL-2 in supernatants were measured by ELISA. Data are presented as average pg/ml ± S.D. Data is derived from three independent experiments (excluding IL-12 which is derived from five experiments). Statistical significance was assessed using unpaired Student's *t* test. (* *p* < 0.05; ** *p* < 0.01).

3.3 DISCUSSION

Dendritic cells are sentinels of the immune system which are not only responsible for directing adaptive immune responses to invading pathogens but also critical for dampening autoimmune responses to self-antigens through the induction of peripheral T cell tolerance (Banchereau et al., 2000; Hawiger et al., 2001; Mellman and Steinman, 2001). The DC-mediated immunological outcome of T cell tolerance versus T cell immunity is highly dependent on the maturation status of the DC and is governed by the expression of co-stimulatory molecules (Steinman and Nussenzweig, 2002; Cools et al., 2007). Indeed, low co-stimulatory molecule-expressing iDCs and high co-stimulatory molecule-expressing mDCs are critical for the induction of T cell tolerance and T cell immunity, respectively (Steinman and Nussenzweig, 2002; Cools et al., 2007). Therefore, regulation of DC maturation status is central to the proper functioning of the immune system, dysregulation of which can result in autoimmunity (Steinman and Nussenzweig, 2002; Decker et al., 2006). It has been identified that DC phenotype and subsequent immune function is highly dependent upon its intracellular redox status (Kantengwa et al., 2003; Chan et al., 2006; Kim et al., 2007). The antioxidant GSH has also been implicated in the regulation of DC immune function (Kim et al., 2007). The main driver of GSH biosynthesis is the transcription factor Nrf2 (Copple, 2012). Nrf2 is emerging as a key player in the regulation of DC co-stimulatory receptor expression and subsequent immune function (Williams et al., 2008; Rangasamy et al., 2010), however, its relationship with basal GSH content and DC immune function has not been established. The present chapter investigated the role of Nrf2 in DC immune function and its relationship with GSH. Previous studies have shown that loss of Nrf2 results in lowered GSH levels in a variety of cell types including hepatocytes, macrophages and fibroblasts (Chan and Kwong, 2000; Kong et al., 2010; Toyama et al., 2011). Although lowered intracellular thiol content has been observed in aged Nrf2 deficient splenic-derived DCs (Kim et al., 2008), reduced cellular GSH levels have not been directly investigated in BMDCs. Our results revealed that Nrf2^{-/-} iDCs exhibited diminished GSH content in relation to their wild type counterpart, a finding which has not yet been established (Aw Yeang et al., 2012). It is established

that loss of Nrf2 results in increased expression of MHCII and molecules CD80 and CD86 in iDCs (Williams et al., 2008; Rangasamy et al., 2010). Our results reaffirm that Nrf2 deficient iDCs exhibit an enhanced maturation phenotype (Aw Yeang et al., 2012). In contrast to this previous study, we also observed a significant increase in the expression of CD40 (Aw Yeang et al., 2012). Reasons for this discrepancy may be due to variation in DC culturing methods e.g. the addition of IL-4 into the DC culture medium as per Rangasamy et al (Rangasamy et al., 2010). Importantly, previous studies looking at the role of Nrf2 in DC immune function were carried out using Nrf2 deficient mice with a different genetic background (Williams et al., 2008; Rangasamy et al., 2010). In support of this, species differences between inbred C57BL/6J and outbred ICR/CD1 mice have been observed within microglial cells inflammatory response to LPS (Nikodemova and Watters, 2011). We sought to determine whether the increase in co-stimulatory receptor expression observed in the Nrf2^{-/-} iDCs was a direct consequence of their lowered GSH levels. By artificially lowering iDC GSH using the GCLC inhibitor BSO, we could not recapitulate the enhanced maturation phenotype seen in the Nrf2^{-/-} iDCs (Aw Yeang et al., 2012). This is in line with previous studies where no differences in co-stimulatory receptor expression were observed between BSO-treated and untreated iDCs (Kuppner et al., 2003; Kamide et al., 2011). We therefore concluded that the increased levels of co-stimulatory molecules displayed in the Nrf2 deficient iDCs was not a consequence of lowered GSH content but was rather due to a cell intrinsic defect.

What other contributing factors could thus be involved in the Nrf2 regulation of co-stimulatory receptor expression in iDCs? The activation of the NF-κB and p38 MAPK intracellular signalling pathways are imperative for the regulation of DC maturation, survival, cytokine secretion and subsequent DC-mediated CD4 effector polarisation (Ardehna et al., 2000; Ouaz et al., 2002; Aiba et al., 2003; Nakahara et al., 2006; Neves et al., 2009; Kim et al., 2010; van de Laar et al., 2010). Studies using Nrf2 deficient mice have illustrated a reciprocal relationship between the Nrf2 and NF-κB transcription factors such that loss of Nrf2 results in enhanced NF-κB activity under oxidative stress conditions (Thimmulappa et al., 2006a; Jin et al., 2008). This transcriptional counter regulation has been observed in other APCs such as

macrophages (Thimmulappa et al., 2006a; Yageta et al., 2011). Curcumin (major component of turmeric spice), which is known to activate Nrf2 (Yang et al., 2009), has been shown to inhibit LPS-induced proinflammatory cytokine secretion in DCs. This was associated with a down regulation in nuclear NF- κ B levels (Cong et al., 2009). Importantly, studies have shown that aged DCs exhibit reduced Nrf2 expression (Kim et al., 2008) and display increased co-stimulatory receptor expression associated with augmented NF- κ B activation in response to self-antigens (Agrawal et al., 2009). Therefore, it would be reasonable to assume that loss of Nrf2 in iDCs could correspond to enhanced nuclear NF- κ B activity, the latter of which contributed to the observed enhanced maturation phenotype. In spite of this, our group found no differences in basal NF- κ B activity between Nrf2^{+/+} and Nrf2^{-/-} iDCs (Aw Yeang et al., 2012). The relationship between Nrf2 and the upstream p38 MAPK is poorly understood. It has been suggested that p38 MAPK phosphorylates Nrf2 which induces an increased association between Nrf2 and Keap-1, ultimately suppressing Nrf2 nuclear translocation in human hepatoma HepG2 cell line (Bryan et al., 2013). The Nrf2 activator sulforaphane inhibits p38 MAPK kinase activity in this model. Furthermore, sulforaphane inhibits p38 MAPK signalling in shear stress activated endothelial cells (Zakkar et al., 2009). Therefore, Nrf2 deficiency may enhance p38 MAPK signalling causing an increase in co-stimulatory receptor expression in iDCs. Recently our group demonstrated that loss of Nrf2 results in increased p38 MAPK phosphorylation in DCs (Al-Huseini et al., 2013). Epigenetic changes in DC gene expression can also influence the DC maturation status and subsequent immune function (Nencioni et al., 2007; Song et al., 2011). Histone acetylation induces changes in gene expression through chromatin expansion, permitting access of transcription factors to the DNA. This process is facilitated by histone acetyltransferase (HAT) enzymes and counter-regulated by histone deacetylase (HDAC) enzymes (Tang et al., 2013). Histone deacetylase activity is essential for the regulation of DC co-stimulatory receptor expression, cytokine secretion, immunostimulatory capacity and maintenance of DC-mediated Th1/Th2 balance (Brogdon et al., 2007; Nencioni et al., 2007; Song et al., 2011). Importantly, it is known that Nrf2 is activated by histone acetylation (Sun et al., 2009). The HAT enzyme CBP involved in the transactivation of Nrf2 has been shown to acetylate

residues contained within the Nrf2 Neh1 ARE DNA-binding region during oxidative stress resulting in the enhancement of the promoter-specific DNA binding capacity of Nrf2 (Sun et al., 2009). Importantly, it has been identified that NF- κ B inhibits the Nrf2 pathway by recruiting the repressive HDAC3 to the ARE which interacts with small Maf K protein and CBP resulting in their deacetylation and functional inhibition, both of which are required for Nrf2 activation (Liu et al., 2008). We have recently shown that the observed increase in co-stimulatory receptor expression in Nrf2^{-/-} iDCs is dependent on HDAC activity as pre-treatment with the HDAC inhibitor valproic acid (VPA) downregulates MHCII and CD86 expression (Aw Yeang et al., 2012). The Nrf2-regulated enzyme HO-1 is involved in the catabolism of cellular heme into biliverdin (Poss and Tonegawa, 1997) and has also been implicated in the regulation of DC co-stimulatory receptor expression and DC cytokine production (Chauveau et al., 2005). Nrf2 deficiency in DCs results in a downregulation of HO-1 gene expression (Rangasamy et al., 2010). Importantly, it has been shown that HO-1 is drastically downregulated upon DC maturation (Chauveau et al., 2005). It is therefore possible that the enhanced maturation phenotype exhibited in the Nrf2^{-/-} iDCs is a result of low HO-1 expression. Heme oxygenase-1 can be marginally induced in Nrf2 deficient cells using the cobalt protoporphyrin (CoPP) (Shan et al., 2006). The effect of pre-treating Nrf2^{-/-} iDCs with CoPP on DC co-stimulatory receptor expression seems worthwhile to examine.

The primary function of iDCs is to continuously acquire antigens from the periphery through numerous endocytic and phagocytic mechanisms (Banchereau et al., 2000). These events are crucial for the induction of T cell immunity and peripheral T cell tolerance. The process of DC maturation is correlated with a down modulation of endo- and phagocytic capacity (Banchereau et al., 2000; Garrett et al., 2000), therefore we hypothesised that increased co-stimulatory receptor expression exhibited in the Nrf2^{-/-} iDCs would be associated with diminished antigen capture capacity. Our results revealed that Nrf2^{-/-} iDCs exhibited a reduced capacity to endocytose dextran and phagocytose both apoptotic and necrotic cells, independently of lowered basal GSH content (Aw Yeang et al., 2012). In contrast, Williams et al. concluded that loss of Nrf2 resulted in enhanced endocytosis of

dextran in resting DCs (Williams et al., 2008). Reasons for this difference may be again due to variation in the mouse strain and DC culturing technique. Loss of endocytic function in maturing DCs is associated with diminished Cdc42 activity (Garrett et al., 2000), levels of Cdc42 expression in Nrf2^{+/+} and Nrf2^{-/-} iDCs could be examined to further delineate the precise role of Nrf2 in DC antigen capture function. Furthermore, it would be noteworthy to see if there are any differences in actin polymerisation between Nrf2^{+/+} and Nrf2^{-/-} iDCs, which is central to DC endocytic and phagocytic mechanisms (Niedergang and Chavrier, 2004). This could be achieved by staining the iDCs undergoing phagocytosis with fluorescently labelled phalloidin (Cougoule et al., 2006). Phalloidin reduces the inorganic phosphate release of polymerizing actin and therefore attaches to the polymerised F-actin, which can be subsequently visualised using fluorescent microscopy (Cougoule et al., 2006). It would also be valuable to compare the migratory capacity of the Nrf2^{+/+} and Nrf2^{-/-} iDCs *in vitro* via a chemotaxis assay, using a chamber transwell plate filter system and a chemoattractant such as chemokine (C-C motif) ligand 19 (CCL19) (Yen et al., 2008).

It would be worthwhile to clarify the role of Nrf2 in DC-antigen acquisition and migratory capacity within an *in vivo* system. This could be achieved by injecting dextran^{FITC}, CFSE-labelled apoptotic or necrotic cells i.v. into the Nrf2^{+/+} and Nrf2^{-/-} mice and examining their lymphoid organs by flow cytometry.

Previous studies have shown that iDCs derived from SLE patients also exhibit defects in mannose-dependent uptake of dextran and phagocytosis of apoptotic cells which correlate with SLE disease activity (Berkun et al., 2008; Monrad et al., 2008). Moreover, SLE iDCs are associated with increased CD86 (Carreno et al., 2009; Gerl et al., 2010), CD40 (Carreno et al., 2009), (Nie et al., 2010) and MHCII (Berkun et al., 2008) expression similar to the findings presented in this chapter. Therefore, it would be valuable to examine the levels of Nrf2 SLE patient-derived DCs relative to healthy controls and to assess whether induction of Nrf2 using CDDO-Me could restore the phagocytic function of DCs in SLE. Importantly, sulforaphane has been shown to restore phagocytic function and enhance bacterial clearance of macrophages from COPD patients (Harvey et al., 2011). Other phenotypic defects

have been observed in SLE-derived DCs including enhanced lymphoid homing CCR7 chemokine levels (Nie et al., 2010), increased ratio of activating (CD32a) to inhibitory (CD32b) FcγR expression (Carreno et al., 2009), decreased DC-SIGN and mannose receptor expression (Monrad et al., 2008). Thus, a detailed phenotypic analysis of Nrf2 deficient DCs in relation to their chemokine, endo- and phagocytic receptor expression is essential to further highlight the connection between Nrf2, DC phenotype and pathogenesis of SLE.

In order for naïve CD8 T cells to become fully activated, they must receive both antigen-specific DC-mediated TCR signals and strong co-stimulatory signals, resulting in the generation of competent effector CTLs and CD8 T cell immunity (Gett et al., 2003). Immature DCs fail to induce CD8 T cell immune responses due to their low co-stimulatory receptor expression (Banchereau et al., 2000). Under resting conditions, iDCs constitutively present self-peptides to CD8 T cells in the periphery. This is imperative for the maintenance of naïve T cell homeostasis as abrogation of interactions of the TCR with MHC:self-peptide complexes presented by iDCs significantly reduces the lifespan of the naïve T cell (Surh and Sprent, 2008). The presence of low level co-stimulatory receptor expression in iDCs enables them to maintain CD8 T cell homeostasis thus limiting overt T cell activation and prevention of detrimental autoimmune responses (Kurts et al., 1997; Steinman and Nussenzweig, 2002). As we have established that Nrf2 deficient iDCs possess enhanced co-stimulatory receptor expression, we hypothesised that this would be associated with an increase in CD8 T cell immunostimulatory capacity in the presence of the antigenic peptide NP68 and importantly the surrogate self-peptide NP34. Using the F5-TCR transgenic antigen-specific murine model, our findings revealed that loss of Nrf2 in iDCs resulted in enhanced iDC-mediated antigen-specific F5 CD8 T cell proliferation and effector function in response to NP68. Examination of other functional readouts of the Nrf2^{+/+} and Nrf2^{-/-} iDCs stimulated F5 CTLs would reaffirm this finding. Cytotoxic T lymphocyte cytolytic capacity could be evaluated using the chromium release assay wherein chromium-labelled target cells release chromium upon CTL-mediated destruction, levels of radioactivity can then be monitored by scintillation counting (Janas et al., 2005). Furthermore,

expression of CTL-released cytolytic molecules such as perforin and granzyme B could also be measured (Janas et al., 2005) by flow cytometry. We have also shown that Nrf2 deficient iDCs induced F5 CD8 T cell proliferation in response to the surrogate self-peptide (Aw Yeang et al., 2012). Taken together, these findings pinpoint an underlying role for Nrf2 in the regulation of iDC-mediated induction of CD8 T cell tolerance. It would be informative to investigate the impact of Nrf2 deficiency in iDCs, in established murine models of tolerance such as the H-Y TCR-transgenic male mice. The male mice contain auto-reactive T cells which express the H-Y TCR that specifically recognise the H-Y self-antigen in the context of MHC H-2D^b. (Maione et al., 2010; Murakami et al., 2010). The consequences of the increased co-stimulatory receptor expression exhibited in the Nrf2^{-/-} iDCs in relation to tolerance could thus be assessed by pre-pulsing the Nrf2^{+/+} and Nrf2^{-/-} iDCs with the H-Y self-antigen *ex vivo*, and injecting them into male transgenic H-Y mice. Signs of enhanced lymphoproliferation within the secondary lymphoid organs could then be examined via flow cytometry.

Dendritic cells can uniquely present exogenous cell-associated antigens derived from phagocytosed dying or infected cells in conjunction with MHCI to naïve CD8 T cells, in a process termed cross-presentation (Joffre et al., 2012). Depending on the context, DC cross-priming mechanisms are essential for the induction of CD8 T cell immunity (e.g. presentation of tumour associated antigens) and maintenance of peripheral CD8 T cell tolerance (e.g. presentation of self-antigens derived from apoptotic cells) (Joffre et al., 2012). We have shown that loss of Nrf2 in iDCs results in an impaired capacity to phagocytose dying cells (Aw Yeang et al., 2012). As phagocytosis is a prelude to the presentation of cell-associated antigens, it is possible that these DCs would be defective in extracting antigen from dying cells and cross-presenting them to naïve CD8 T cells. Conversely, our findings have illustrated that the LPS-activated Nrf2^{-/-} DCs exhibited an enhanced capacity to stimulate F5 CD8 T cells, following extraction of cognate antigen from necrotic cells in relation to their wild type counterpart. The phagocytosis of exogenous material results in the formation of the phagosome (Trombetta and Mellman, 2005). During cross-presentation, this vacuolar compartment is responsible for transporting its

cargo to the proteasome for proteolytic degradation into antigenic peptides, followed by MHCI peptide loading within the ER and translocation to the surface of the DC for presentation to naïve CD8 T cells (Trombetta and Mellman, 2005). The efficiency of the cross-presentation is highly dependent on the DC phagosomal pH (Kotsias et al., 2013). Unlike other phagocytes which contain acidic, proteolytically active phagosomes, DCs possess more alkaline (at around pH 7.5), proteolytically inactive, vacuolar compartments ensuring that the exogenous material is not completely degraded and can be efficiently loaded onto MHCI molecules (Joffre et al., 2012). Alkalisiation of the DC phagosome is induced by increased NOX2-dependent ROS production within the phagosome (Kotsias et al., 2013). Defects in ROS levels in the phagosome have resulted in impaired DC-mediated cross-presentation of cell-associated antigens to CD8 T cells (Joffre et al., 2012). We and others have shown that Nrf2 deficient DCs exhibit enhanced intracellular ROS levels in comparison to their wild type control (Williams et al., 2008; Aw Yeang et al., 2012). Therefore, if increased ROS in the phagosome amounts to increased antigen cross-presentation, this may explain why Nrf2 deficient DCs exhibited an increased cross-priming capacity. However, increased total ROS production in DCs does not necessarily correlate with increased DC phagosomal ROS production as demonstrated in Savina et al. 2009 (Savina et al., 2009). Evaluation of DC phagosomal ROS production could be achieved by examining Nrf2^{+/+} and Nrf2^{-/-} iDCs phagocytosis of latex beads labelled with the ROS indicator dihydrorhodamine (Savina et al., 2009). This dye fluoresces green when oxidised and changes in ROS can be analysed by flow cytometry (Savina et al., 2009). It has been shown that efficient DC cross-presentation, NOX2 assembly and ROS production within the phagosome is dependent upon the activity of the small rho GTPase Rac2 (Savina et al., 2009). It would be interesting to look at the levels of Rac2 in the phagosomes by intracellular staining and flow cytometric analysis (Savina et al., 2009). This experiment is not without its limitations; however, as we cannot say for certain that the observed increase in naïve F5 CD8 T cell proliferation is exclusively due to increased cross-presentation capacity. It could be attributable to differences in co-stimulatory receptor expression induced by LPS and necrotic cells between both genotypes and/or increases in DC cytokine production such as IL-12, which

promotes CD8 T cell activation and proliferation. It would be valuable to assess co-stimulatory receptor expression and IL-12 production in Nrf2^{+/+} and Nrf2^{-/-} DCs treated with both LPS and necrotic cells to clarify the role of Nrf2 in DC cross-presentation.

Dendritic cells are not only responsible for the initiation of T cell immune responses, they are essential for influencing the nature of the response elicited. Functional differentiation of T cells into effector T cells, is highly dependent upon their exposure to cytokines within their microenvironment, that are derived from the DC itself or other innate immune cells at the site of infection (Curtsinger et al., 1999). Dendritic cells secrete an array of cytokines depending upon the type of pathogen initially encountered by the DC, the corresponding PRR signalling and resultant upregulation of cytokine gene expression (Geijtenbeek and Gringhuis, 2009; Kawai and Akira, 2011). For example, *E. coli*-derived LPS is known to signal through TLR4 on the DC, inducing the production of IFN γ and IL-12 which results in CD4 Th1 type immune responses (Agrawal et al., 2003). In contrast, Schistosome helminth infection stimulates TLR2 signalling, resulting in the production of IL-4 by DCs thus driving a Th2 response (van der Kleij et al., 2002). Dendritic cell-mediated regulation of Th1/Th2 differentiation is highly dependent upon its intracellular redox status (Ohtani et al., 2005; Traidl-Hoffmann et al., 2005; Vassallo et al., 2005; Chan et al., 2006; Csillag et al., 2010). It has been established that loss of Nrf2 in DCs perturbs their intracellular redox status (Aw Yeang et al., 2012), which has been associated with a dysregulation of CD4 effector Th1/Th2 differentiation toward the Th2 phenotype under oxidative stress conditions (Williams et al., 2008). We therefore sought to investigate the impact of loss of Nrf2 on basal and LPS-induced DC Th1 TNF α , IL-12p70 and Th2 IL-2 cytokine secretion. Results revealed that upon LPS activation, Th1 TNF α and IL-12p70 levels were decreased in the Nrf2 deficient DCs and in relation to their wild type counterpart. However no differences in IL-2 production were observed between the genotypes. Moreover Th2 cytokines IL-13 and IL-5 were undetected within this LPS-stimulated context. This suggests that Nrf2 regulates DC Th1 cytokine production in response to a DC-mediated Th1 polarising stimuli. It has been previously shown that Nrf2 deficient DCs derived

from an ICR/CD1 background exhibited enhanced basal TNF α levels, a trend which was accentuated upon activation with the allergen ragweed extract (Rangasamy et al., 2010). A previous study has shown that Nrf2 deficient DCs secrete significantly enhanced basal IL-12p40 levels in relation to their wild type (Rangasamy et al., 2010). Moreover, LPS-treated Nrf2^{+/+} and Nrf2^{-/-} DCs derived from a similar C57BL/6J background to the presented work produced similar levels of IL-12p40 production (Chan et al., 2006). However, as IL-12p70 levels have been examined in the present chapter these reports are not comparable. It has been established that loss of Nrf2 in DCs results in increased Th1 IL-12 and Th2 IL-13 production in relation to their wild type control in response to ambient particulate matter (Williams et al., 2008). Although both Th1 and Th2 cytokines were induced, the level of Th2 IL-13 production in relation to Th1 IL-12 production was increased indicating an intense pro-Th2 bias (Williams et al., 2008). From the presented work we cannot conclude that loss of Nrf2 results in a skewed Th1/Th2 cytokine profile towards a Th2 dominance. However, we can still ascertain that Nrf2 plays a pivotal role in the regulation of DC Th1 cytokine production, therefore suggesting that Nrf2 plays a role in maintaining the DC Th1/Th2 cytokine secretion. It is well recognised that LPS induces a strong Th1 response (Agrawal et al., 2003), therefore it would not have been a suitable stimulus to examine the effects of loss of Nrf2 on Th2 cytokine production. Other reports which have demonstrated the loss of Nrf2 in DCs results in a skewing towards the Th2 phenotype were based on DCs exposed to a Th2 inducing allergen (Williams et al., 2008; Rangasamy et al., 2010). It is therefore necessary to stimulate the Nrf2^{+/+} and Nrf2^{-/-} DCs with TLR2 agonists such as Pam3Cys-Ser-Lys4 (pam3cys) or Schistosoma egg antigens (SEA) which are known to induce a strong DC Th2 cytokine profile (Agrawal et al., 2003) to confirm that Nrf2 regulates DC Th1/Th2 cytokine production within a Th2 inducing context. It would also be informative to look at the role of Nrf2 in DC secretion of other CD4 lineage-specific polarising cytokines e.g. TGF β , IL-6 and IL-23 for Th17 differentiation (Mangan et al., 2006) to gain a detailed cytokine profile of the Nrf2 deficient DCs.

In conclusion, the present chapter revealed a novel role of Nrf2 in the GSH-independent regulation of co-stimulatory receptor expression, antigen capture function and antigen-specific CD8 T cell stimulatory capacity in bone marrow-derived iDCs. Importantly, our results implicate a role of Nrf2 in the maintenance DC-mediated peripheral CD8 T cell tolerance. Moreover, this study highlighted the role of Nrf2 in regulating DC-mediated Th1 cytokine production ultimately influencing the nature of the immune response elicited.

CHAPTER FOUR

THE ROLE OF NRF2 IN T CELL IMMUNE FUNCTION

4.1 INTRODUCTION

It is evident from the previous chapter that the redox sensitive transcription factor Nrf2 is a key player in the regulation of DC immune function (Aw Yeang et al., 2012). However, the role of Nrf2 in T cell immune function is poorly understood. We therefore sought to explore the impact of Nrf2 in T cells. It is recognised that Nrf2 maintains Th1/Th2 balance within an oxidative stress environment (Rangasamy et al., 2005; Williams et al., 2008). However, its precise role in T cell development, T cell activation and CD4 differentiation remains to be fully elucidated. The hypothesis of the work described in this chapter is that similarly to DCs, Nrf2 regulates T cell immune function. To test this hypothesis, we utilised the Nrf2 deficient mouse experimental system, to examine the role of Nrf2 in T cell development, its impact on naïve T cell redox homeostasis, T cell activation and proliferative capacity, and its influence on CD4 T cell polarisation.

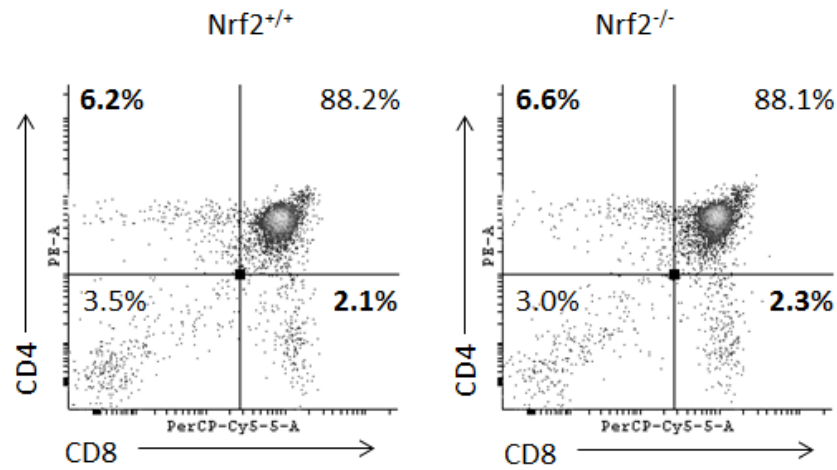
4.2 RESULTS

4.2.1 Loss of Nrf2 does not affect T cell development

Nrf2 is a master regulator of an array of cytoprotective and antioxidant defence genes in response to oxidative insult (Copple et al., 2010). Perturbation of the Nrf2 pathway results in enhanced oxidative stress in a variety of immune cells including DCs and macrophages (Thimmulappa et al., 2006b; Williams et al., 2008), illustrating the important role of Nrf2 in the maintenance of cellular redox homeostasis. It has been established that increased mitochondrial oxidative stress induces apoptosis in the thymus, resulting in aberrant thymic CD4 and CD8 T cell populations (Case et al., 2011). This suggests that oxidative stress disrupts T cell development in the thymus. However, it is not known whether loss of Nrf2 affects the composition of CD4:CD8 T cell populations within this region and ultimately T cell development. In order to address this, freshly isolated thymocytes derived from Nrf2^{+/+} and Nrf2^{-/-} mice were incubated with fluorescently labelled CD4^{PE} and CD8^{TC} cell surface antibodies and analysed by flow cytometry. Flow cytometric analysis revealed distinctive subpopulations of T cells at different stages of development including CD4⁻CD8⁻ DN cells, CD4⁺CD8⁺ DP cells, and mature CD4⁺ and CD8⁺ single

positive (SP) T cells (**Figure 4.1A**, representative dot plot). Percentages of the aforementioned T cell populations, as denoted within the quadrant regions in the dot plots (**Figure 4.1A**), were comparatively similar within the thymus of both Nrf2^{+/+} and Nrf2^{-/-} mice, as quantified in **Figure 4.1B** (Nrf2^{+/+} versus Nrf2^{-/-}, CD4⁻CD8⁻: 2.9 ± 0.3 % vs. 2.8 ± 0.3 %; CD4⁺CD8⁺: 87.3 ± 0.6 % vs. 88.8 ± 0.8 %; CD4⁺: 7.0 ± 0.3 % vs. 6.0 ± 0.4 %; CD8⁺: 2.8 ± 0.5 % vs. 2.4 ± 0.4 %). The 2:1 ratio of CD4 SP cells to CD8 SP cells observed in both mice (**Figure 4.1B**, Nrf2^{+/+} 7.0% : 2.8% versus Nrf2^{-/-} 6% : 2.4%) is characteristic of murine C57BL/6J thymocytes (Kontgen et al., 1993). Overall, this indicates that Nrf2 is not required for competent T cell development within the thymus.

A



B

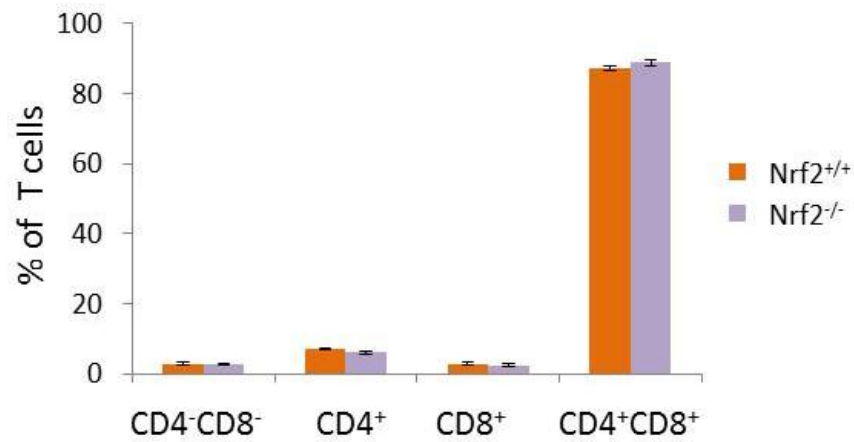
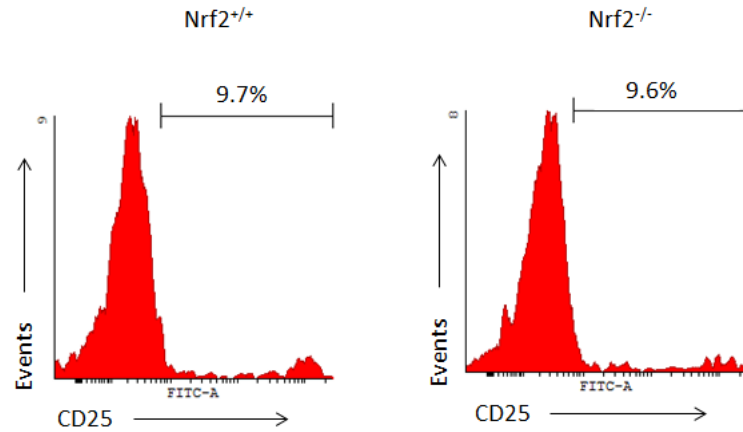


Figure 4.1 Nrf2 does not regulate T cell development within the thymus. Isolated thymocytes derived from 10-12 week old Nrf2^{+/+} and Nrf2^{-/-} mice were stained with CD4^{PE} and CD8^{TC} antibodies and analysed by flow cytometry. **A.** Representative flow scatter dot plot diagrams of murine Nrf2^{+/+} (left) and Nrf2^{-/-} (right) thymocytes for expression of CD4 and CD8. Percentages of T cell populations are indicated within the quadrants. **B.** Quantification of percentage of CD4⁻CD8⁻, CD4⁺, CD8⁺ and CD4⁺CD8⁺ T cells within Nrf2^{+/+} (orange) and Nrf2^{-/-} (purple) thymocytes. Data are presented as average T cell percentages \pm S.E.M. Data are derived from six independent experiments.

4.2.2 Loss of Nrf2 does not influence nTreg populations within the thymus

Within the thymus, a distinctive immunosuppressive CD4 T cell subtype arises from mature CD4 SP thymocytes, namely nTregs (Sakaguchi, 2004). Natural occurring T regulatory cells are responsible for suppressing the activation and proliferation of peripheral self-reactive T cells, which have managed to escape negative selective events in the thymus. Therefore, they are critical for the dampening of autoimmune responses and subsequent disease (Sakaguchi, 2004). The development and immune function of nTregs are highly dependent upon the transcriptional activity of FoxP3 and expression of the IL-2 receptor α chain (IL-2R α) CD25, both of which can be used as suitable markers for nTreg identification within the thymus (Yagi et al., 2004). It is recognised that Nrf2 deficient mice develop multi-organ autoimmune inflammation and lymphoproliferation (Ma et al., 2006). This suggests that there may be a potential defect in the nTreg population and subsequent immunosuppressive function. Although Nrf2 did not alter mature CD4 T cell proportions within the thymus, it does not exclude potential defects in nTreg development. In order to decipher whether Nrf2 regulated nTreg development within the thymus, Nrf2^{+/+} and Nrf2^{-/-} thymocytes were incubated with fluorescently labelled CD4^{PE}, CD8^{TC} and CD25^{Alexafluor®488} cell surface antibodies and analysed by flow cytometry. Cells were gated on the CD8⁻CD4⁺ T cell subset (upper left quadrant section) within the dot plot (**Figure 4.1A**). The CD25 expression was determined within this population and presented as a histogram (**Figure 4.2A**). These results indicate that there were no differences in CD25 expression between Nrf2^{+/+} and Nrf2^{-/-} mature CD4 SP T cells (**Figure 4.2B**, $11 \pm 1.1\%$ versus $9.6 \pm 1.4\%$). This indicates that Nrf2 does not regulate nTreg subpopulations within the thymus.

A



B

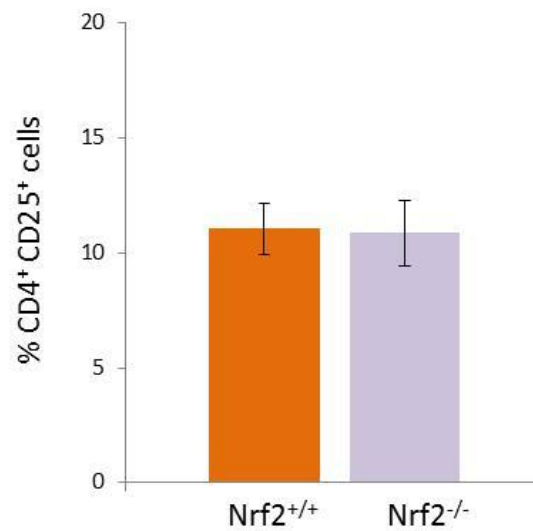


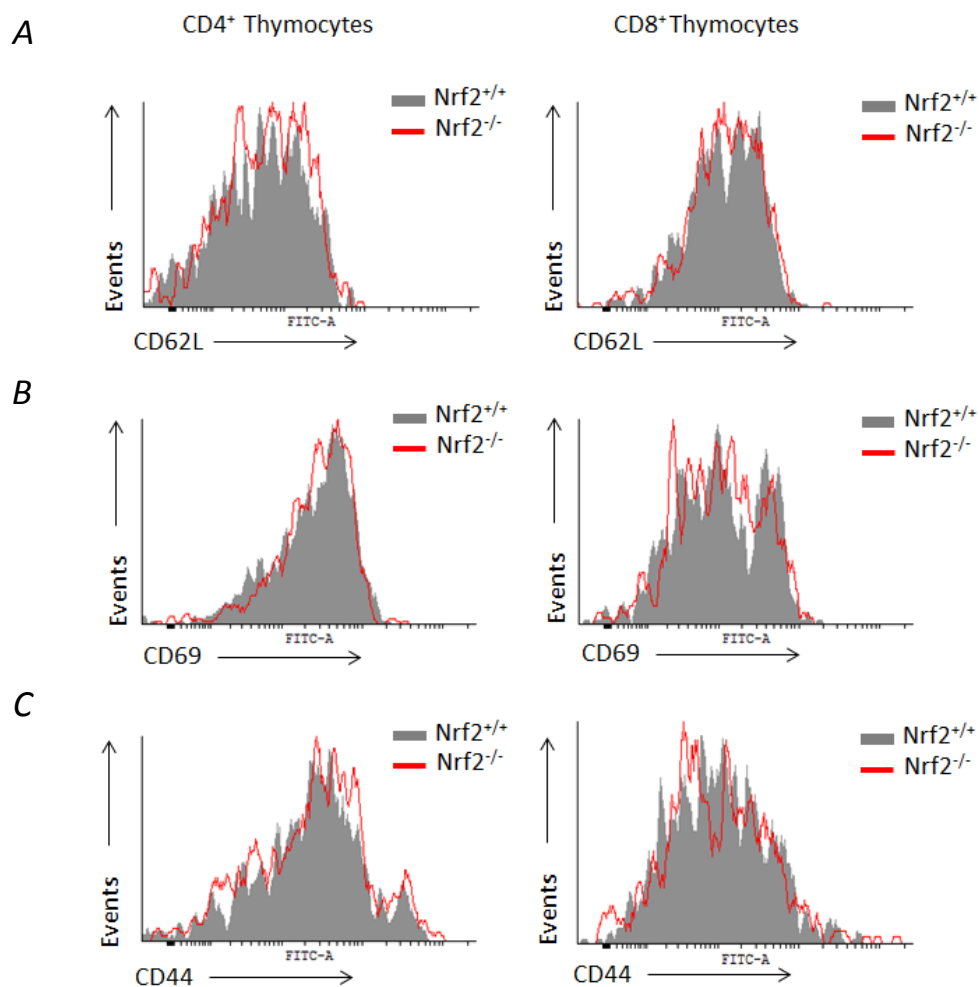
Figure 4.2 Nrf2 does not influence natural occurring T regulatory cell populations within the thymus. Isolated thymocytes derived from 10-12 week old $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice were stained with $CD4^{PE}$, $CD8^{TC}$ and $CD25^{Alexafluor^{488}}$ antibodies and analysed by flow cytometry. Cells were gated on $CD4^{+}$ T cell population for analysis **A**. Representative Histograms of $Nrf2^{+/+}$ (left) and $Nrf2^{-/-}$ (right) thymocytes for expression of CD25. The percentages of $CD4^{+}$ T cells expressing high levels of CD25 are indicated within the marker **B**. Quantification of percentage of $CD4^{+}$ T cells expressing high levels of CD25 ($CD4^{+}CD25^{+}$) in $Nrf2^{+/+}$ (orange) and $Nrf2^{-/-}$ (purple) thymocytes. Data are presented as average percentage of $CD4^{+}CD25^{+}$ T cells \pm S.E.M. Data are derived from four independent experiments.

4.2.3 Loss of Nrf2 does not influence CD62L, CD69 and CD44 expression within the thymus

As SP T cells mature within the thymic medulla region of the thymus, their chemokine and cell surface molecule expression changes. This reflects their ability to migrate through the thymic medulla, ultimately leading to their emigration from the thymus, through the blood stream to peripheral lymphoid organs (Nakayama et al., 2002). Phenotypic maturation of SP T cells is associated with increased expression of the lymphoid homing receptor CD62L (L-selectin) (Rosen et al., 2003) and decreased expression of the early T cell activation marker CD69 (Rosen et al., 2003). Overexpression of CD69 has been shown to result in reduced T cell numbers in peripheral lymphoid organs (Nakayama et al., 2002). The cell surface glycoprotein CD44 is expressed on progenitor cells enabling their migration to the thymus (Wu et al., 1993). It is down regulated during CD4⁻CD8⁻ DN1-DN4 differentiation, but is reacquired at CD4⁺CD8⁺ DP development (Rajasagi et al., 2009). Single positive T cells regain high expression of CD44 (Rajasagi et al., 2009). However, it is not required for egression from the thymus; but rather it operates in a similar way as in peripheral T cells, wherein it supports T cell expansion in conjunction with TCR activation. Therefore it can be seen as a T cell activation marker (Foger et al., 2000; Rajasagi et al., 2009).

We sought to investigate whether Nrf2 may play a role in the regulation of CD62L, CD69 and CD44 in SP CD4 and CD8 T cells within the thymus; therefore, implicating a role in thymic lymphoid homing capacity and activation status. To address this issue, Nrf2^{+/+} and Nrf2^{-/-} thymocytes were incubated with combinations of fluorescently labelled CD4^{PE}, CD8^{TC}, CD62L^{FITC}, CD69^{FITC} and CD44^{FITC} cell surface antibodies and analysed by flow cytometry. Cells were gated on the CD8⁻CD4⁺ (upper left quadrant section) or CD8⁺CD4⁻ (lower right quadrant section) T cell subsets for CD4⁺ thymocytes and CD8⁺ thymocytes, respectively (**Figure 4.1A**). Expression of CD62L (**Figure 4.3A**), CD69 (**Figure 4.3B**) and CD44 (**Figure 4.3C**) were determined within these gated populations and presented as overlay histograms. Up regulation or down modulation of the cell surface molecules were identified through a rightward or leftward shift in the histogram peak, respectively. Results

portrayed that there were no differences in CD62L (**Figure 4.3A**) or CD69 (**Figure 4.3B**) expression in either CD4 (left hand panel) or CD8 (right hand panel) SP thymic T cells between Nrf2^{+/+} and Nrf2^{-/-} mice. Collated data for cell surface molecule expression is depicted in **Figure 4.3D**. There was a slight increase in Nrf2^{-/-} CD4⁺ CD44 expression in relation to its wild type counterpart (**Figure 4.3C** left hand panel CD4⁺ thymocytes, mean fluorescence intensity (MFI) 5466.3 versus 6134.7). However, no differences in CD44 expression were observed within the SP CD8 population (**Figure 4.3C** right hand panel CD8⁺ thymocytes). Taken together, this suggests that Nrf2 does not play a role in the regulation of CD62L and CD69 expression in mature thymic T cells, thus excluding Nrf2 from regulating particular aspects of mature thymic lymphoid homing capacity. Furthermore, this transcription factor plays a negligible role in the regulation of CD44 expression in mature thymic T cells.



D

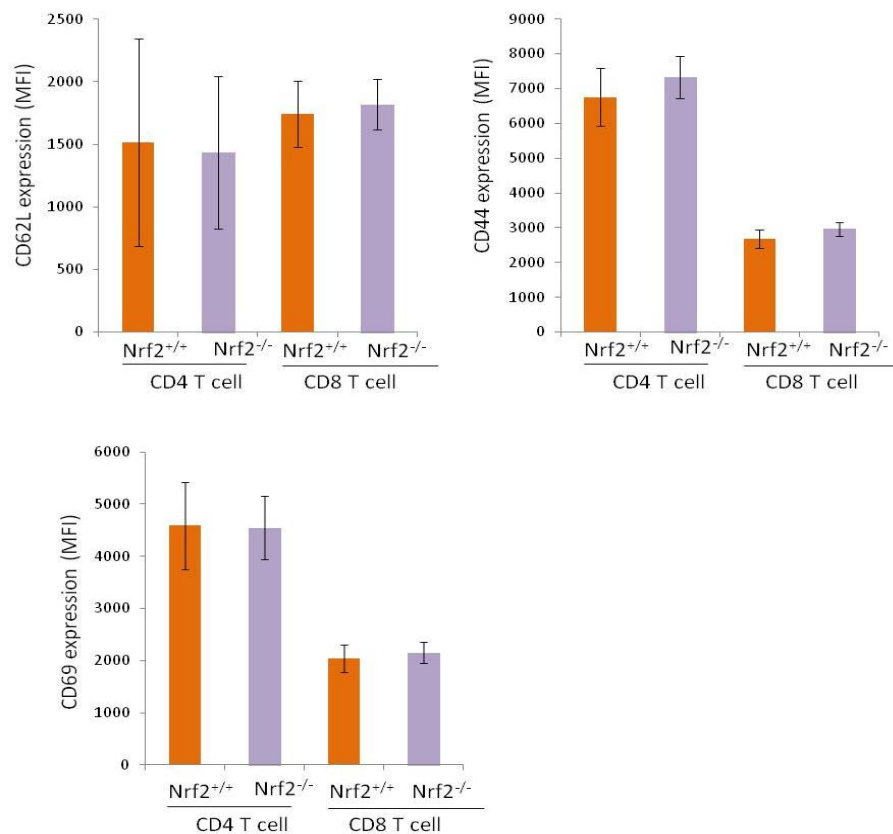


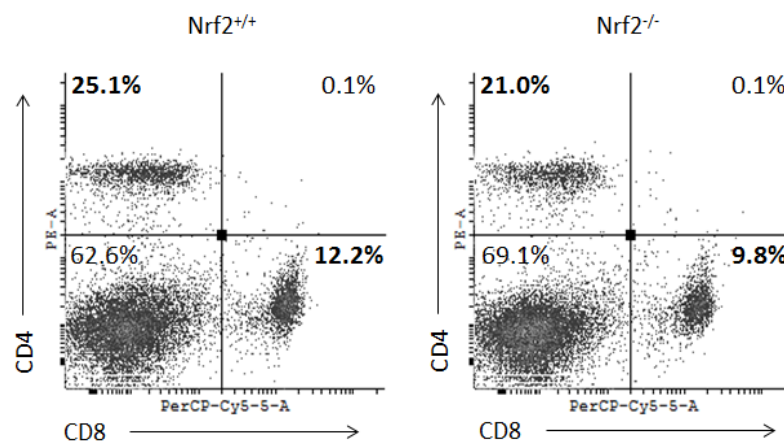
Figure 4.3 Nrf2 does not regulate single positive CD4 and CD8 T cell expression of CD62L, CD69 or CD44 cell surface markers within the thymus. Isolated thymocytes derived from 10-12 week old Nrf2^{+/+} and Nrf2^{-/-} mice were stained with CD4^{PE} and CD8^{TC} in conjunction with CD62L^{FITC}, CD69^{FITC} or CD44^{FITC} antibodies and analysed by flow cytometry. Cells were gated on CD4⁺ T cell (left) or CD8⁺ T cell (right) populations for analysis. Representative overlay histograms of Nrf2^{+/+} (grey block) and Nrf2^{-/-} (red line) for thymocytes for **A.** CD62L, **B.** CD69 and **C.** CD44 expression. Data are representative of 3 independent experiments.. **D.** Collated CD62L, CD44 and CD69 expression graphs are presented as mean fluorescence intensity (MFI) \pm S.D.

4.2.4 Loss of Nrf2 does not affect T cell populations in peripheral lymphoid organs.

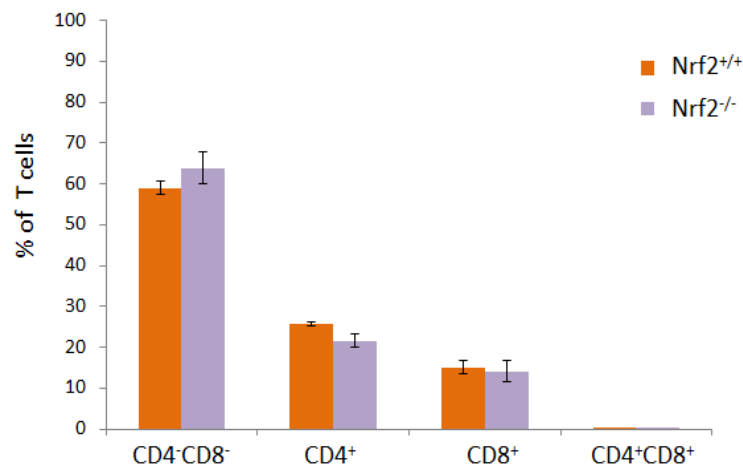
We subsequently investigated whether loss of Nrf2 had any impact on CD4 and CD8 T cell proportions within secondary lymphoid organs such as the spleen and lymph node. To address this, newly isolated Nrf2^{+/+} and Nrf2^{-/-} splenic T lymphocytes were incubated with fluorescently labelled CD4^{PE} and CD8^{TC} cell surface antibodies and analysed by flow cytometry. Results revealed that there were no differences in the

composition of CD4 and CD8 T cell between Nrf2^{+/+} and Nrf2^{-/-} T cells within the spleen, as illustrated in the representative dot plot (**Figure 4.4Ai**) and quantified in **Figure 4.4Aii** (Nrf2^{+/+} - CD4⁺ 25.7 ± 0.47%, CD8⁺ 15.2 ± 1.5% versus Nrf2^{-/-} - CD4⁺ 21.7 ± 1.7%, CD8⁺ 14.1 ± 2.5%). Similar results were attained for Nrf2^{+/+} and Nrf2^{-/-} T cells within the lymph node (Representative dot plot **Figure 4.4Bi**, collated data **Figure 4.4Bii** Nrf2^{+/+} - CD4⁺ 42.2 ± 1.5%, CD8⁺ 29.3 ± 1.8% versus Nrf2^{-/-} - CD4⁺ 40.2 ± 0.9%, CD8⁺ 28.2 ± 1.5%). Conclusively, this illustrates that Nrf2 plays a negligible role in the homeostasis of T cell subpopulations within secondary lymphoid organs.

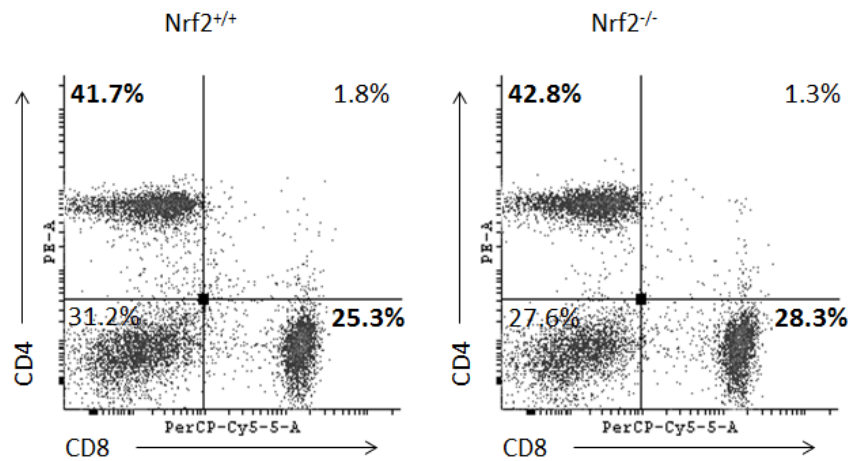
Ai



Aii



Bi



Bii

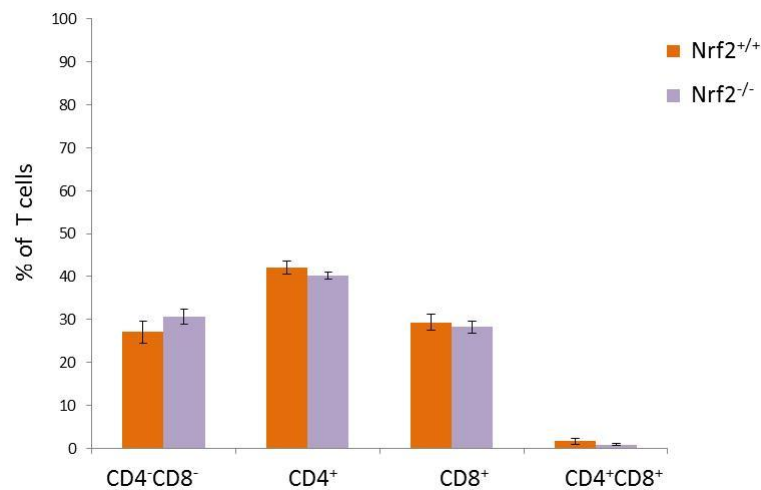
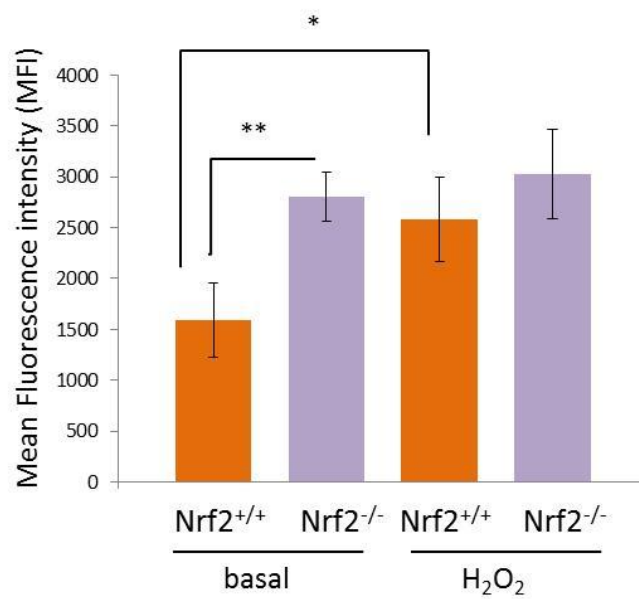


Figure 4.4 Nrf2 does not regulate the composition of CD4 and CD8 T cells within the periphery. Isolated murine Nrf2^{+/+} and Nrf2^{-/-} splenocytes and lymph node-derived T cells were stained with CD4^{PE} and CD8^{TC} antibodies and analysed by flow cytometry. Representative flow scatter dot plot diagrams of Nrf2^{+/+} (left) and Nrf2^{-/-} (right) **Ai.** splenocytes and **Bii.** lymph node-derived T cells for expression of CD4 and CD8. Percentages of T cell populations are indicated within the quadrants. Quantification of percentage of CD4⁺CD8⁻, CD4⁺, CD8⁺ and CD4⁺CD8⁺ T cells in Nrf2^{+/+} (orange) and Nrf2^{-/-} (purple) **Aii.** splenocytes and **Bii.** lymph node-derived T cells. Data are presented as average T cell percentages ± S.E.M. Data are derived from four independent experiments.

4.2.5 Loss of Nrf2 results in elevated intracellular ROS levels in peripheral naïve T cells

It is well known that Nrf2 regulates intracellular ROS levels in a variety of cell types including DCs and macrophages (Thimmulappa et al., 2006b; Williams et al., 2008); however, its role in the regulation of peripheral naïve T cell intracellular redox homeostasis has yet to be established. We therefore examined the intracellular ROS levels both basally and in response to the oxidative stress inducer H₂O₂, in freshly isolated splenic T cells derived from Nrf2^{+/+} and Nrf2^{-/-} mice, using the fluorescent redox-sensitive probe, Dihydroethidium (DHE). Dihydroethidium is a colourless substance that can freely diffuse across the cell membrane. It is oxidised by intracellular superoxide radicals, emitting a red fluorescence upon oxidation. Therefore, it serves as a suitable ROS detector for flow cytometric analysis (Burnaugh et al., 2007). As demonstrated in **Figure 4.5**, naïve Nrf2^{-/-} T cells displayed significantly higher ROS levels in comparison to their wild type control (1595.8 ± 366.1 versus 2805.5 ± 244.1 , $p = 0.01$). Reactive oxygen species in the Nrf2^{-/-} T cells were slightly higher than the H₂O₂-treated Nrf2^{+/+} T cells, although this was not significant (2805.5 ± 244.1 versus 2581.2 ± 412.9). Moreover, there was a significant increase in ROS levels between basal and H₂O₂ treated Nrf2^{+/+} T cells (1595.8 ± 366.1 versus 2581.2 ± 412.9 , $P = 0.03$). Taken together, this suggests that Nrf2 plays a role in the regulation of intracellular redox homeostasis in peripheral naïve T cells.



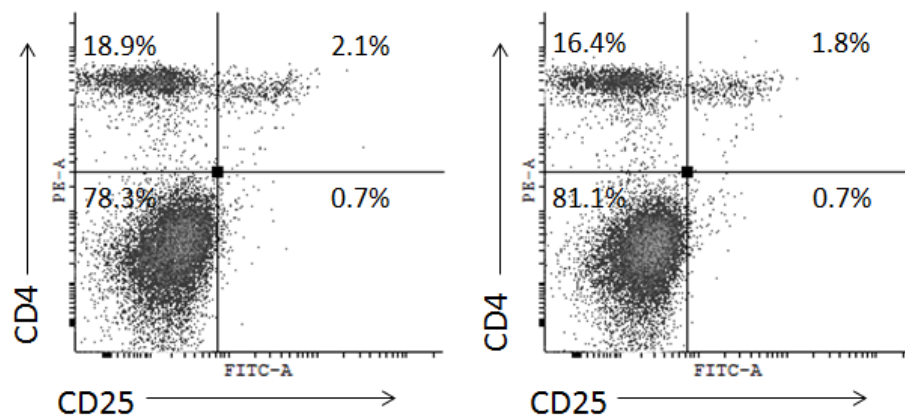
4.5 Nrf2 regulates intracellular ROS levels in naïve T cells. Isolated splenocytes from 10-12 week old Nrf2^{+/+} (orange) and Nrf2^{-/-} (purple) mice were treated with or without H₂O₂ (basal) for 10 min. Cells were subsequently incubated with the ROS indicator dihydroethidium and analysed by flow cytometry. Data are presented as average mean fluorescence intensity \pm S.D. Statistical significance was assessed using unpaired Student's *t* test. Data are representative of four independent experiments. (*, *p* < 0.05; **, *p* < 0.01).

4.2.6 Loss of Nrf2 does not influence peripheral CD4 T cell activation with respect to CD25 expression

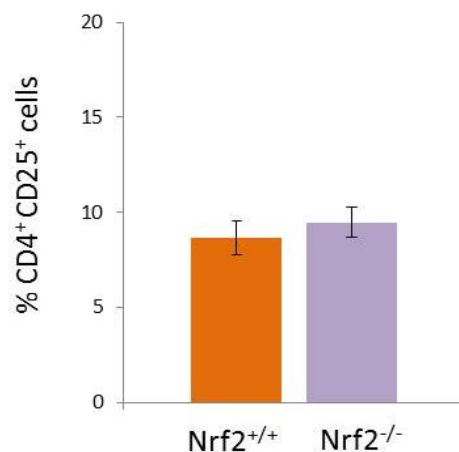
During antigen-specific T cell activation, the high affinity IL-2R subunit CD25 (also known as IL-2R α) is upregulated; combining with the IL-2R β (CD122) and γ_c (CD132) chains to complete the multimeric high affinity IL-2R on the T cell surface (Gaffen, 2001). Activated T cell secretion of IL-2 then works in an autocrine fashion by binding to the high affinity IL-2R, driving the clonal expansion and differentiation of naïve antigen-specific T cells into effector T cells (Boyman and Sprent, 2012). As a result, CD25 expression is regarded as one of the early markers of T cell activation (Gundelach et al., 2013). We investigated whether increased ROS present in the Nrf2 deficient T cells may support T cell activation, thus enhancing CD25 expression in naïve CD4 T cells within secondary lymphoid organs. To achieve this, Nrf2^{+/+} and Nrf2^{-/-} splenic T lymphocytes were incubated with fluorescently labelled CD4^{PE} and

CD25^{Alexafluor®488} cell surface antibodies and analysed by flow cytometry. Results revealed that there were no differences in percentages of CD4⁺CD25⁺ T cells (upper right quadrant section) between Nrf2^{+/+} and Nrf2^{-/-} T cells within the spleen; as illustrated in **Figure 4.6Ai** and further quantified in **Figure 4.6Aii** ($8.7 \pm 0.9\%$ versus $9.5 \pm 0.8\%$). Similarly, there were no changes in naïve CD4 T cell CD25 expression between Nrf2^{+/+} and Nrf2^{-/-} mice within the lymph node (Dot plot **Figure 4.6Bi** and collated data **Figure 4.6Bii** $9.3 \pm 1.0\%$ versus $10.9 \pm 0.7\%$). In summary, these results suggest that Nrf2 does not regulate CD25 expression in naïve CD4 T cells in secondary lymphoid organs.

Ai



Aii



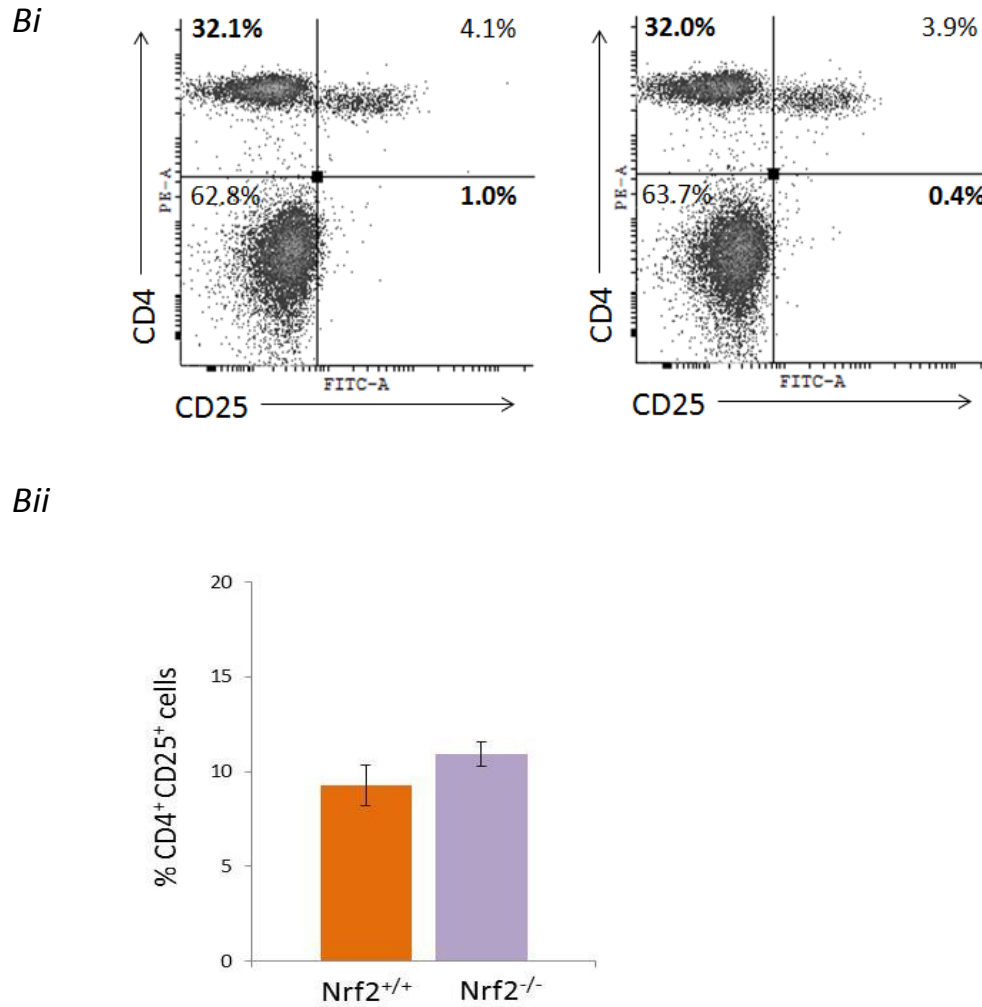


Figure 4.6 Nrf2 does not regulate the CD4 expression of CD25 activation marker within the periphery. Isolated murine Nrf2^{+/+} and Nrf2^{-/-} splenocytes and lymph node derived T cells were stained with CD4^{PE} and CD25^{Alexafluor®488} antibodies and analysed by flow cytometry. Cells were gated on CD4⁺ T cell population for analysis. Representative flow scatter dot plot diagrams of Nrf2^{+/+} (left) and Nrf2^{-/-} (right) **Ai.** splenocytes and **Bi.** lymph node-derived T cells for expression of CD4 and CD25. Percentages of T cell populations are indicated within the quadrants. Quantification of percentage of CD4⁺CD25⁺ T cells in Nrf2^{+/+} (orange) and Nrf2^{-/-} (purple) **Aii.** splenocytes and **Bii.** lymph node-derived T cells. Data are presented as average percentage of CD4⁺CD25⁺ T cells ± S.E.M. Data are derived from four independent experiments.

4.2.7 Naïve Nrf2 deficient T cells manifest low levels of T cell activation within lymphoid organs.

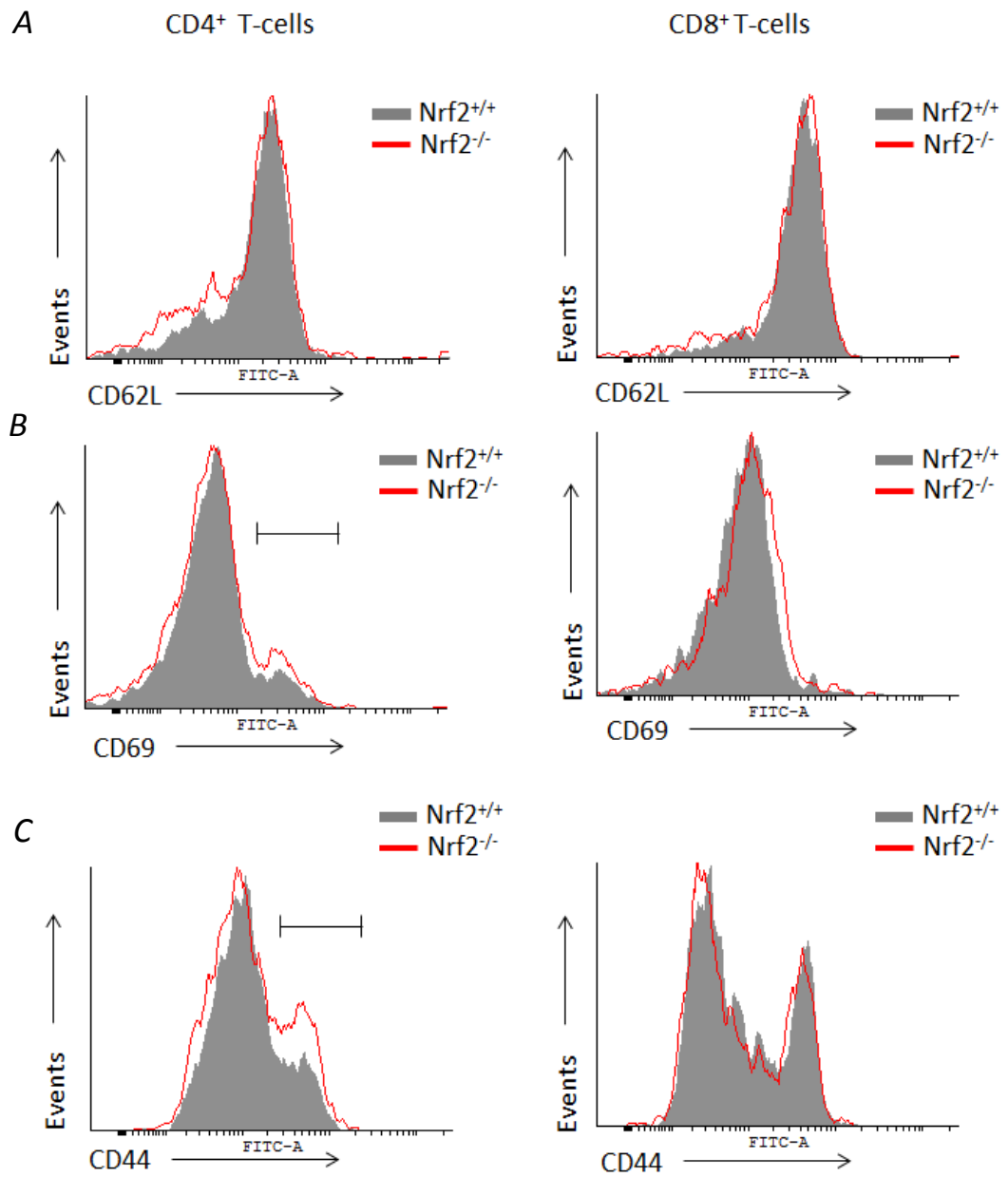
As a consequence of T cell activation, the patterns of chemokines and other activation molecules are transformed on the T cell surface. This facilitates further T cell proliferation, differentiation and egression from the lymphoid organs to sites of

infection (Masopust and Schenkel, 2013). In addition to heightened CD25 expression, T cell activation results in an upregulation of CD69 and CD44 (Foger et al., 2000). In contrast to the thymus, CD69 is seen as the earliest T cell activation marker, rapidly induced from as early as 1 hour post-T cell activation and is involved in the promotion of T cell proliferation (Marzio et al., 1999). Similarly, CD44 supports signal transduction through the TCR/CD3 complex (Foger et al., 2000). T cell activation is also associated with a down regulation in CD62L expression, with an increase in levels of non-lymphoid markers such as CXCR3, thus permitting departure from the lymphoid organs (Masopust and Schenkel, 2013). Oxidative stress can enhance and down regulate CD44 and CD62L expression in peripheral naïve T cells, respectively (Case et al., 2011; Foster et al., 2013). However it is unknown whether the increased ROS levels exhibited in the Nrf2 deficient T cells would promote their T cell activation status through regulation of CD44, CD69 and CD62L expression in secondary lymphoid organs. To examine this possibility, Nrf2^{+/+} and Nrf2^{-/-} splenocytes (**Figure 4.7A-C**) and lymphocytes (**Figure 4.7D-E**) were incubated with combinations of fluorescently labelled CD4^{PE}, CD8^{TC}, CD62L^{FITC}, CD69^{FITC} and CD44^{FITC} cell surface antibodies and analysed by flow cytometry. Cells were gated on the CD8⁻CD4⁺ (upper left quadrant section) or CD8⁺CD4⁻ (lower right quadrant section) T cell subsets shown in **Figure 4.5Ai** for spleen and **Figure 4.5Bi** for lymph node. Expression of CD62L (**Figure 4.7A and D**), CD69 (**Figure 4.7B and E**) and CD44 (**Figure 4.7C and F**) were determined within these gated populations and presented as overlay histograms. Up regulation or down modulation of the cell surface molecules were identified through a rightward or leftward shift in the histogram peak, respectively. Changes in higher expressing cell populations were also monitored.

With reference to the spleen, no differences in CD62L expression were exhibited between Nrf2^{+/+} and Nrf2^{-/-} CD4 or CD8 T cells (**Figure 4.7A** left hand panel, CD4 T cells; right hand panel, CD8 T cells). Similarly, no changes in CD69 expression were observed between splenic Nrf2^{+/+} and Nrf2^{-/-} CD4 T cells (**Figure 4.7B** left hand panel, CD4⁺ T cells). Although there appears to be a shift in population from the low expressing CD69 peak to the higher expressing CD69 peak in Nrf2^{-/-} CD4 T cells

(highlighted by marker), there was no major difference in the percentage of high expressing CD69 cells between both groups (**Figure 4.7B** left hand panel, CD4⁺ T cells, Nrf2^{+/+} 10.8% versus Nrf2^{-/-} 13.3%). In contrast, Nrf2^{-/-} CD8 T cells appeared to express higher levels of CD69 in comparison to their wild type counterpart as seen in the rightward histogram peak shift in **Figure 4.7B** (right hand panel, Nrf2^{+/+} MFI 1158.9 versus Nrf2^{-/-} MFI 1303.34). As indicated in **Figure 4.7C**, Nrf2^{-/-} CD4 T cells displayed greater levels of CD44 expression in relation to the control group (Nrf2^{+/+} MFI 1998.7 versus Nrf2^{-/-} MFI 2321.5). Although a rightward shift in histogram peak was not observed, there was population change in the proportion of Nrf2^{-/-} CD4 T cells expressing higher levels CD44 (as indicated by the marker) in comparison to the wild type counterpart (20.4% versus 27.3%). Similar expression of CD44 was observed between Nrf2^{+/+} and Nrf2^{-/-} splenic CD8 T cells (**Figure 4.7C** left hand panel). Our results demonstrate that loss of Nrf2 results in marginal low levels of CD4 T cell activation in terms of enhanced CD44 expression within the spleen.

With reference to the lymph node, both naïve Nrf2^{-/-} CD4 and CD8 T cells exhibited a down regulation in CD62L expression in comparison to their wild type counterparts, as observed as a leftward peak shift illustrated in **Figure 4.7D** (Left hand panel CD4⁺ T cells, Nrf2^{+/+} MFI 2423.9 versus Nrf2^{-/-} MFI 2033.5; right hand panel CD8⁺ T cells Nrf2^{+/+} MFI 3818.37 versus Nrf2^{-/-} MFI 3280.7). In contrast, no changes in CD69 expression were observed between Nrf2^{+/+} and Nrf2^{-/-} lymph node-derived CD4 and CD8 T cells (**Figure 4.7E** left and right hand panel, respectively). Lymph node-derived naïve Nrf2^{-/-} CD4 T cells displayed enhanced CD44 expression in relation to their wild type control group (**Figure 4.7F** left hand panel CD4⁺ T cells Nrf2^{+/+} MFI 7783.4, percentage of CD4⁺CD44^{hi} cells 8.7% versus Nrf2^{-/-} MFI 11231.4, 13.3%). This proportional increase was also apparent in Nrf2^{-/-} CD8 T cells (**Figure 4.7F** left hand panel CD4⁺ T cells, Nrf2^{+/+} MFI 7783.4, percentage of CD4⁺CD44^{hi} cells 8.7% versus Nrf2^{-/-} MFI 11231.4, 13.3%). Taken together our results demonstrate that loss of Nrf2 results in marginally low levels of CD4 and CD8 T cell within the lymph node regions of secondary lymphoid organs.



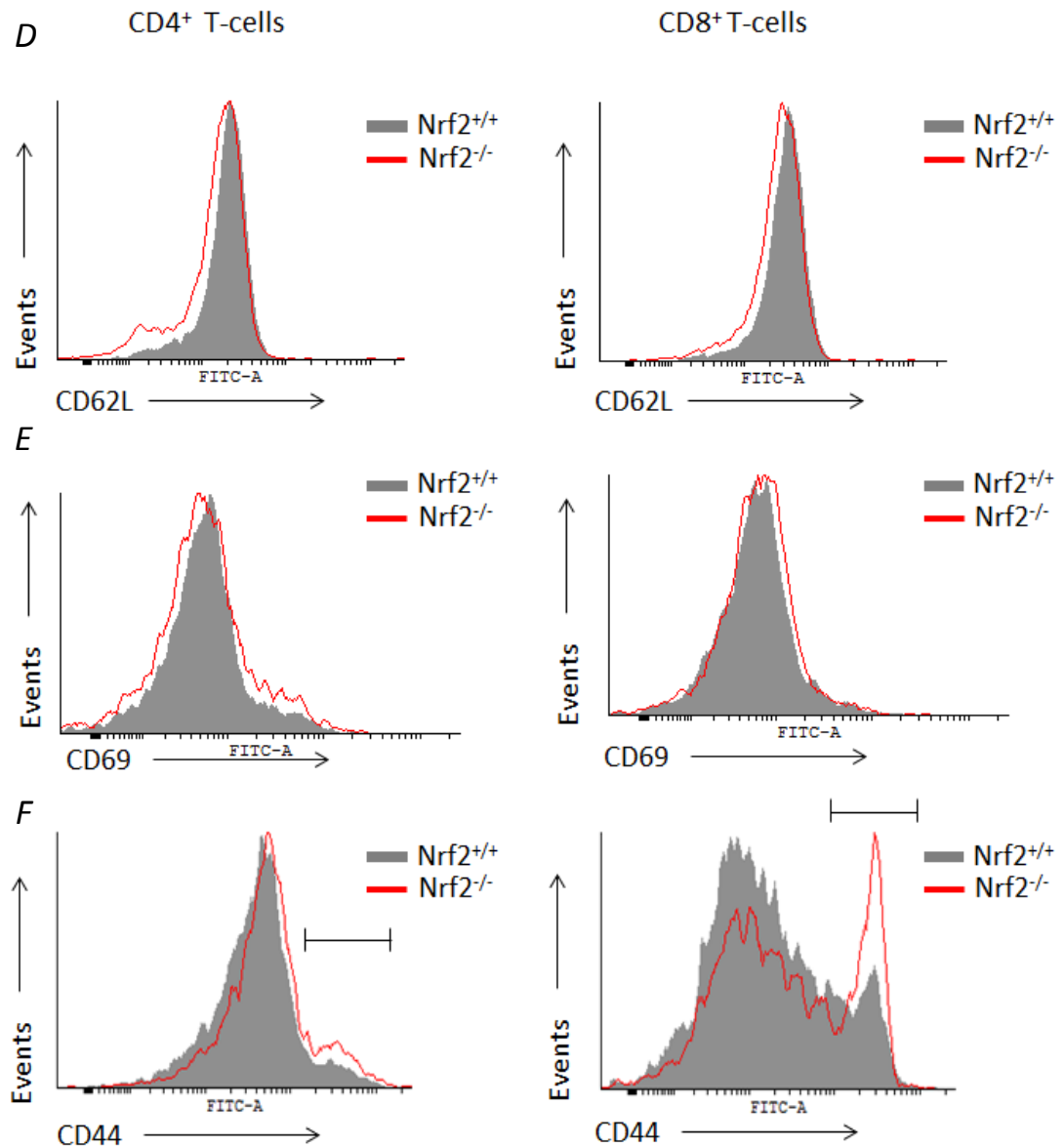


Figure 4.7 Loss of Nrf2 results in the induction of low level naïve T cell activation. Isolated splenocytes and lymph node derived T cells derived from 10-12 week old *Nrf2*^{+/+} and *Nrf2*^{-/-} mice were stained with CD4^{PE} and CD8^{TC} in conjunction with CD62L^{FITC}, CD69^{FITC} or CD44^{FITC} antibodies and analysed by flow cytometry. Cells were gated on CD4⁺ T cell (left) or CD8⁺ T cell (right) populations for analysis. Representative overlay histograms of *Nrf2*^{+/+} (grey block) and *Nrf2*^{-/-} (red line) splenocytes for **A.** CD62L, **B.** CD69 and **C.** CD44 expression and lymph node derive T cell expression of **D.** CD62L, **E.** CD69 and **F.** CD44. Markers indicated above histogram peaks denote high cell surface marker expressing populations. Data are representative of 3 independent experiments

4.2.8 Loss of Nrf2 results in marginally enhanced effector T cell proliferation.

We have previously shown that Nrf2 deficient naïve T cells elicit signs of low level T cell activation in the secondary lymphoid organs. In light of this finding, we investigated whether loss of Nrf2 would influence the proliferative capacity of peripheral naïve splenic T cells in response to non-specific TCR/CD3 stimulation, using increasing concentrations of immobilised CD3 and CD28 antibodies. Utilisation of the CD3 antibody facilitates the cross linkage of the TCR/CD3 complex enabling non-specific T cell proliferation in the absence of APC-mediated antigen stimulation (Harding et al., 1992). Similarly to APC-mediated T cell activation, additional co-stimulatory signalling is necessary for competent T cell proliferation (Harding et al., 1992). Therefore, in addition to TCR/CD3 complex signal transduction, CD28 antibody is provided, which crosslinks with its co-stimulatory molecule, enabling optimal non-specific T cell proliferation (Harding et al., 1992). Results revealed that there were no significant differences in T cell proliferation between Nrf2^{+/+} and Nrf2^{-/-} splenic T cells in response to increasing concentrations of plate bound anti-CD3 and anti-CD28 (**Figure 4.8A**).

Nrf2 plays a pivotal role in the regulation of naïve T cell differentiation in a murine asthma model (Rangasamy et al., 2005). However, it is unknown whether Nrf2 influences the proliferative capabilities of effector T cells in response to increasing concentrations of immobilised anti-CD3 and anti-CD28 under normal conditions. To address this, naïve Nrf2^{+/+} and Nrf2^{-/-} splenic T cells were stimulated with plate bound anti-CD3 (2 µg/ml) and anti-CD28 (1 µg/ml). Cells were cultured for 6 days to enable differentiation into effector T cells which occurs 4-5 days post-naïve T cell activation (Murphy et al., 2012). Day 6 splenic effector Nrf2^{+/+} and Nrf2^{-/-} T cells were re-stimulated with increasing concentrations of plate bound anti-CD3 and anti-CD28 as above. Results demonstrated that Nrf2^{-/-} T cells exhibited significantly enhanced T cell proliferation in relation to the wild type control at all concentrations of anti-CD3 and anti-CD28 (**Figure 4.8B**). At the 0.25 µg/ml CD3/CD28 concentration there was an approximate 23% increase in T cell proliferation in effector Nrf2^{-/-} T cells in comparison to their wild type counterpart (**Figure 4.8B**, Nrf2^{+/+} $6.4 \times 10^4 \pm 3931$ cpm versus Nrf2^{-/-} $7.9 \times 10^4 \pm 5811$ cpm, $p =$

0.01). An approximate 27% and 56% increase were also observed at the 0.5 $\mu\text{g/ml}$ ($\text{Nrf2}^{+/+}$ $6.6 \times 10^4 \pm 3734$ cpm versus $\text{Nrf2}^{-/-}$ $8.6 \times 10^4 \pm 6122$ cpm, $p = 0.01$) and 1 $\mu\text{g/ml}$ ($\text{Nrf2}^{+/+}$ $5.7 \times 10^4 \pm 4867$ cpm versus $\text{Nrf2}^{-/-}$ $8.9 \times 10^4 \pm 13744$ cpm, $p = 0.02$) concentrations of anti-CD3 and anti-CD28, respectively. This result suggests that Nrf2 plays a role in the regulation of effector T cell proliferation.

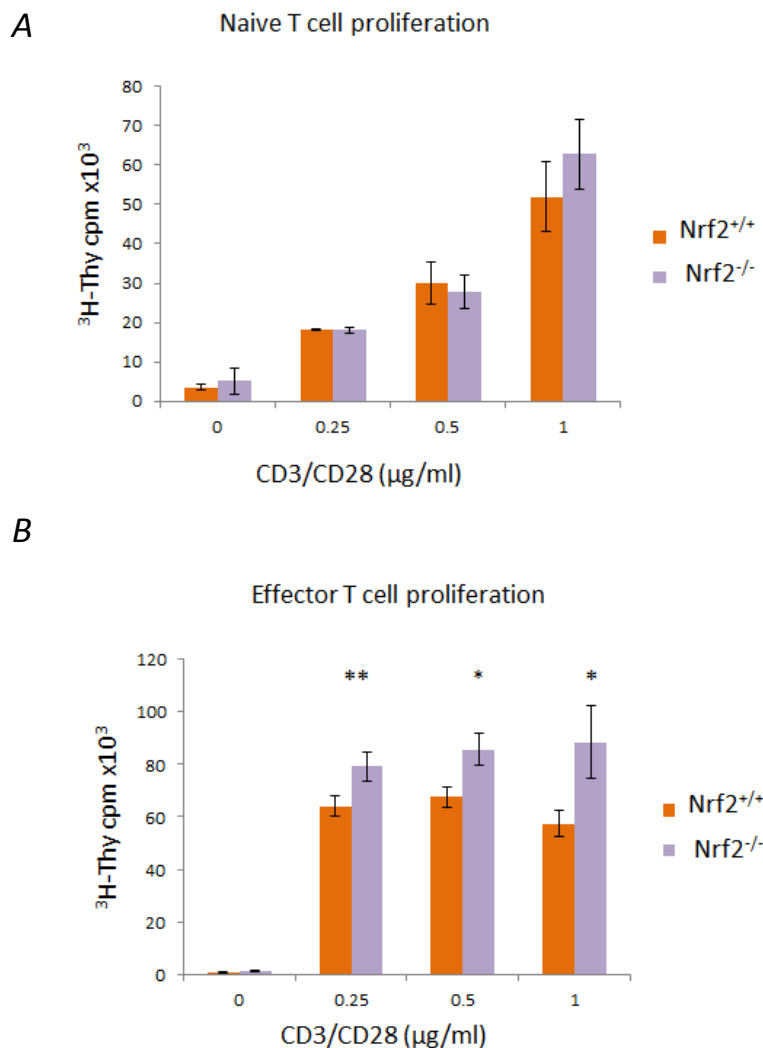
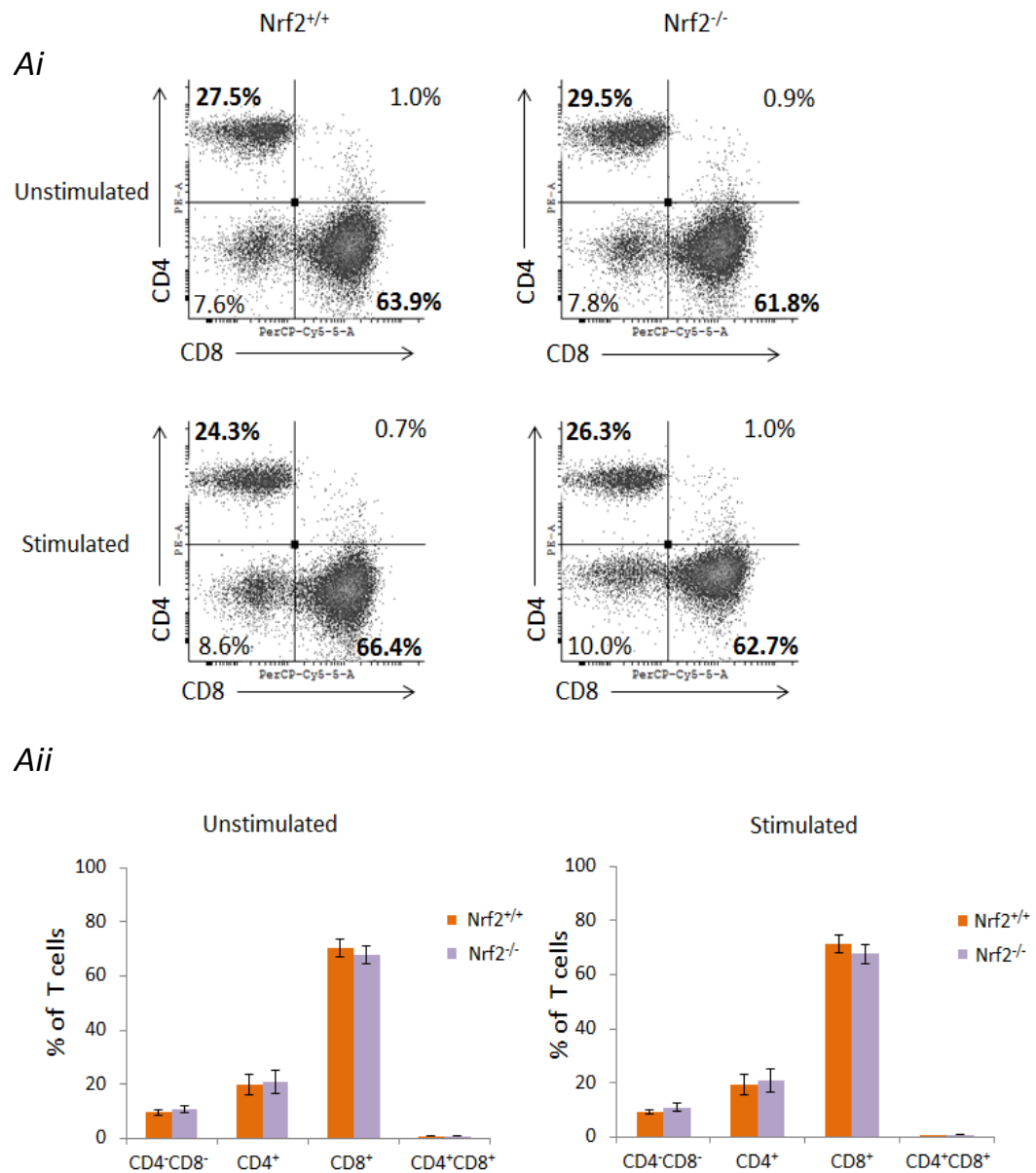


Figure 4.8 Loss of Nrf2 results in the induction enhanced effector T cell proliferation in response to TCR stimulation. **A.** Naïve splenic T cells and **B.** Day 6 cultured effector T cells derived from 10-12 week old $\text{Nrf2}^{+/+}$ and $\text{Nrf2}^{-/-}$ mice were stimulated with increasing concentrations of immobilised anti-CD3 and anti-CD28 antibodies for 72 h. [^3H]-Thymidine ($^3\text{H-Thy}$) was added for the last 16 h. Proliferation of T cells was determined by scintillation counting of incorporated $^3\text{H-Thy}$. Data are presented as average $^3\text{H-Thy}$ scintillation counts \pm S.D. Statistical significance was assessed using unpaired Student's t test. Data are representative of four independent experiments. (* $p < 0.05$; ** $p < 0.01$).

4.2.9 Loss of Nrf2 results in enhanced effector CD4 T cell IFN γ production.

Nrf2 regulates CD4 T cell production of Th2 IL-4 and IL-13 in response to antibody re-stimulation in a murine model of asthma (Rangasamy et al., 2005). Furthermore, Nrf2 regulates CD4 T cell Th1 IFN γ production under hypoxic conditions (Roman et al., 2010). Therefore, Nrf2 plays a pivotal role in the maintenance of CD4 T cell differentiation within these settings. We thus endeavoured to examine the effects of loss of Nrf2 on effector CD4 T cell and CD8 T cell immune function by measuring IFN γ production under normal conditions. This was achieved by re-stimulating day 6 splenic Nrf2^{+/+} and Nrf2^{-/-} T cells with or without immobilised anti-CD3 and anti-CD28 for 5 hours. Cells were subsequently incubated with fluorescently labelled CD4^{PE} and CD8^{TC}, followed by cell fixation and permeabilisation, and finally intracellular cytokine staining with fluorescently labelled IFN γ ^{FITC}. Effector T cells were then analysed using flow cytometry. Initially, we examined whether loss of Nrf2 had any impact on the proportion of effector CD4 and CD8 splenic T cells with or without antibody re-stimulation. Results portrayed that there were no differences in the composition of CD4 and CD8 T cells between effector Nrf2^{+/+} and Nrf2^{-/-} splenic T cells in the presence and absence of antibody stimulation (**Figure 4.9Ai**, representative dot plot; **Figure 4.9Aii**, collated data). It is worthy to note that CD3 and CD28 T cell stimulation induces the preferential expansion of CD8 T cells over CD4 T cells (Ernst et al., 1989). As a result, the splenic CD4:CD8 T cell ratio which was originally approximately 2:1 for naïve T cells (**Figure 4.5Ai**), has reversed in splenic effector T cells in favour of the CD8 subtype at approximately 1:3 ratio (**Figure 4.9Ai and ii**). This was similar for both unstimulated and re-stimulated effector T cells (**Figure 4.9Ai and ii**). Subsequently, cells were gated on the CD8⁻CD4⁺ T cell subset (**Figure 4.9Ai**, lower panel, upper left quadrant section) and CD8⁺CD4⁻ T cell subset (**Figure 4.9Ai**, lower panel, bottom right quadrant section) within the dot plot to assess CD4 and CD8 T cell effector IFN γ production as illustrated in **Figure 4.9B**. The IFN γ expression was determined within these populations (**Figure 4.9B**). Results demonstrated that loss of Nrf2 gave rise to a significant increase in the percentage of CD4⁺IFN γ ⁺ effector T cells in comparison to their wild type control (**Figure 4.9Bi**, Nrf2^{+/+} 4.4 \pm 0.6% versus Nrf2^{-/-} 8.6 \pm 1.2%, $p = 0.02$) which is

indicative of enhanced CD4 Th1 T cells (Hsieh et al., 1993). In contrast, there were no significant differences in CD8⁺IFN γ ⁺ T cell percentages between Nrf2^{+/+} and Nrf2^{-/-} mice (**Figure 4.9Bii**). Taken together, this suggests that loss of Nrf2 results in enhanced effector CD4 T cell IFN γ production.



B

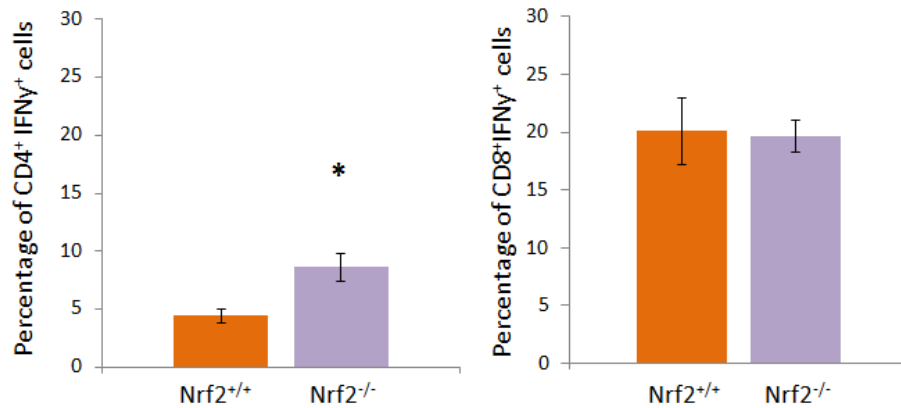


Figure 4.9 Loss of Nrf2 enhances the proportion of CD4 effector IFN γ producing T cells during re-stimulation. Nrf2^{+/+} and Nrf2^{-/-} day 6 splenic effector T cells were re-stimulated with or without immobilised CD3 and CD28 antibodies and assessed for intracellular cytokine IFN γ production in CD4⁺ and CD8⁺ T cell populations. **Ai.** Representative flow scatter dot plot diagrams of Nrf2^{+/+} (left) and Nrf2^{-/-} (right) effector splenic effector CD4 and CD8 T cell populations. Percentages of T cell populations are indicated within the quadrants. **Aii.** Quantification of percentages of CD4⁺ CD8⁺, CD4⁺ CD8⁻ and CD4⁻ CD8⁺ T cells in Nrf2^{+/+} (orange) and Nrf2^{-/-} (purple) in presence (right) or absence (left) of antibody stimulation. **B.** Quantified percentages of CD4⁺ IFN γ ⁺ (left) (gated on CD4⁺ high population) and CD8⁺ IFN γ ⁺ cells (gated on CD8⁺ high population) (right) in Nrf2^{+/+} (orange) and Nrf2^{-/-} (purple) splenic effector T cells. Data are presented as average percentage of T cells \pm S.E.M. Statistical significance was assessed using unpaired Student's *t* test. Data are derived from four independent experiments (*, *p* < 0.05).

4.2.10 Nrf2 regulates CD4 effector subset differentiation in purified splenic CD4 T cells

As we have identified a key role of Nrf2 in the maintenance of CD4 Th1 effector T cell immune function, we subsequently investigated whether loss of Nrf2 had an impact on the production of other CD4 lineage specific cytokines including TNF α (Th1), IL-5 (Th2) and IL-17 (Th17), in an attempt to further explore the role of Nrf2 in the regulation of CD4 subset differentiation (Zhu et al., 2010). In order to address this, purified day 6 Nrf2^{+/+} and Nrf2^{-/-} splenic effector CD4 T cells were re-stimulated with plate bound anti-CD3 and anti-CD28 for 16 hours and cytokine production in the effector T cell supernatants were assessed by ELISA method. Initially to reaffirm our previous finding that loss of Nrf2 enhances IFN γ production in CD4 T cells, we examined the differences in absolute amounts (pg/ml) of IFN γ between the two mice. Results confirmed that effector CD4 Nrf2^{-/-} T cells secreted

significantly elevated levels of IFN γ in comparison to the wild type control (**Figure 4.10A**, Nrf2^{+/+} 87704.3 \pm 2930.0 pg/ml versus Nrf2^{-/-} 113151.9 \pm 7724.7 pg/ml, p = 0.04). In contrast, Nrf2 deficient T cells produced lower levels of TNF α with respect to the wild type effector T cells (**Figure 4.10B**, Nrf2^{+/+} 1898.7 \pm 113.6 pg/ml versus Nrf2^{-/-} 1297.1 \pm 7.6 pg/ml, p = 0.04). Studies have shown that loss of Nrf2 does not influence naïve CD4 Th2 cytokine secretion in response to antibody stimulation (Rangasamy et al., 2005); however, it is unknown whether loss of Nrf2 impacts on CD4 effector T cell Th2 cytokine secretion. We therefore assessed the secretion of Th2 specific IL-5 production between re-stimulated effector Nrf2^{+/+} and Nrf2^{-/-} CD4 T cells. Results illustrated that Nrf2^{-/-} splenic effector CD4 T cells secreted significantly less IL-5 in comparison to the wild type control (**Figure 4.10C**, Nrf2^{+/+} 875.5 \pm 51.7 pg/ml versus Nrf2^{-/-} 716.9 \pm 31.2 pg/ml, p = 0.04). The recently identified CD4 Th17 subset plays a pivotal role in the pathogenesis of autoimmune diseases such as MS (Zhu and Qian, 2012). Therefore we examined whether Nrf2 had an impact on effector Th17 cytokine production of IL-17A under normal conditions. Results clearly demonstrated that Nrf2 deficient effector CD4 T cell secreted significantly increased levels of IL-17A in comparison to effector Nrf2^{-/-} CD4 T cells (**Figure 4.10D**, Nrf2^{+/+} 263.3 \pm 104.7 pg/ml versus Nrf2^{-/-} 693.8 \pm 131.0 pg/ml, p = 0.01). Taken together our results reveal a role for Nrf2 in the control of CD4 effector Th1/Th2 cell differentiation. Importantly, results have indicated a novel underlying role of Nrf2 in the regulation of the effector T cell IL-17 production.

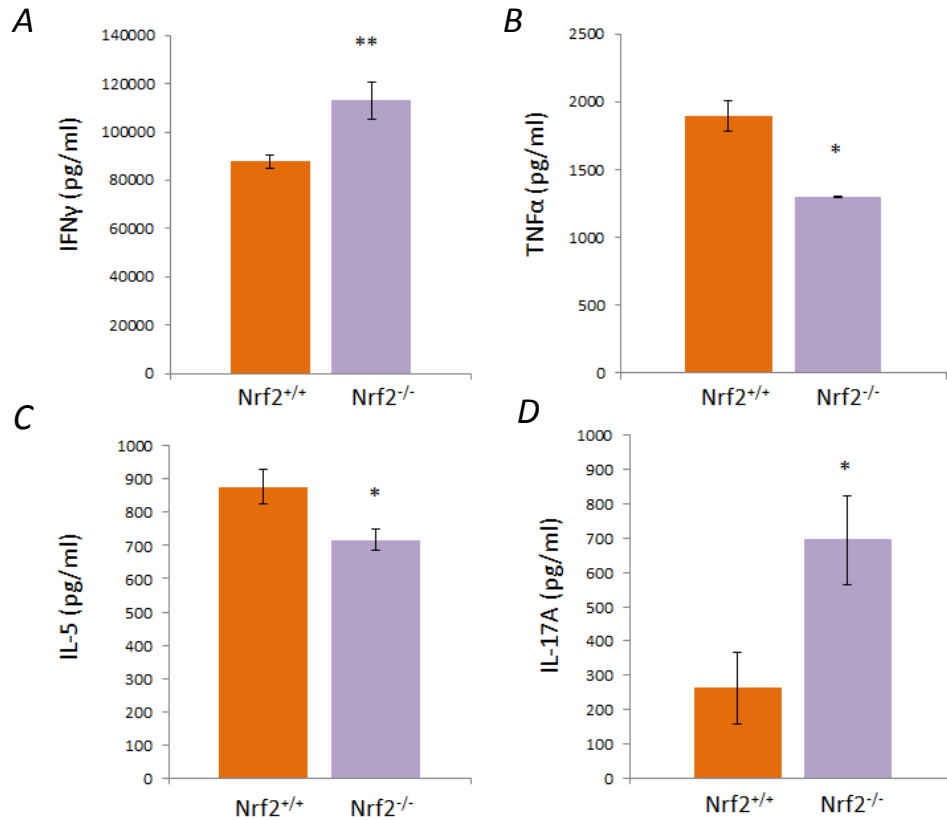


Figure 4.10 Nrf2 regulates CD4 effector T cell signature cytokine production. CD4 purified day 6 cultured Nrf2^{+/+} and Nrf2^{-/-} effector T cells were re-stimulated with immobilised anti-CD3 and anti-CD28 antibodies for 24 h. Levels of **A.** IFNγ, **B.** TNFα, **C.** IL-5 and **D.** IL-17A in supernatants were measured by ELISA. Data are presented as average pg/ml ± S.D. Data is representative of three independent experiments. Statistical significance was assessed using unpaired Student's *t* test. (* *p* < 0.05; ** *p* < 0.01).

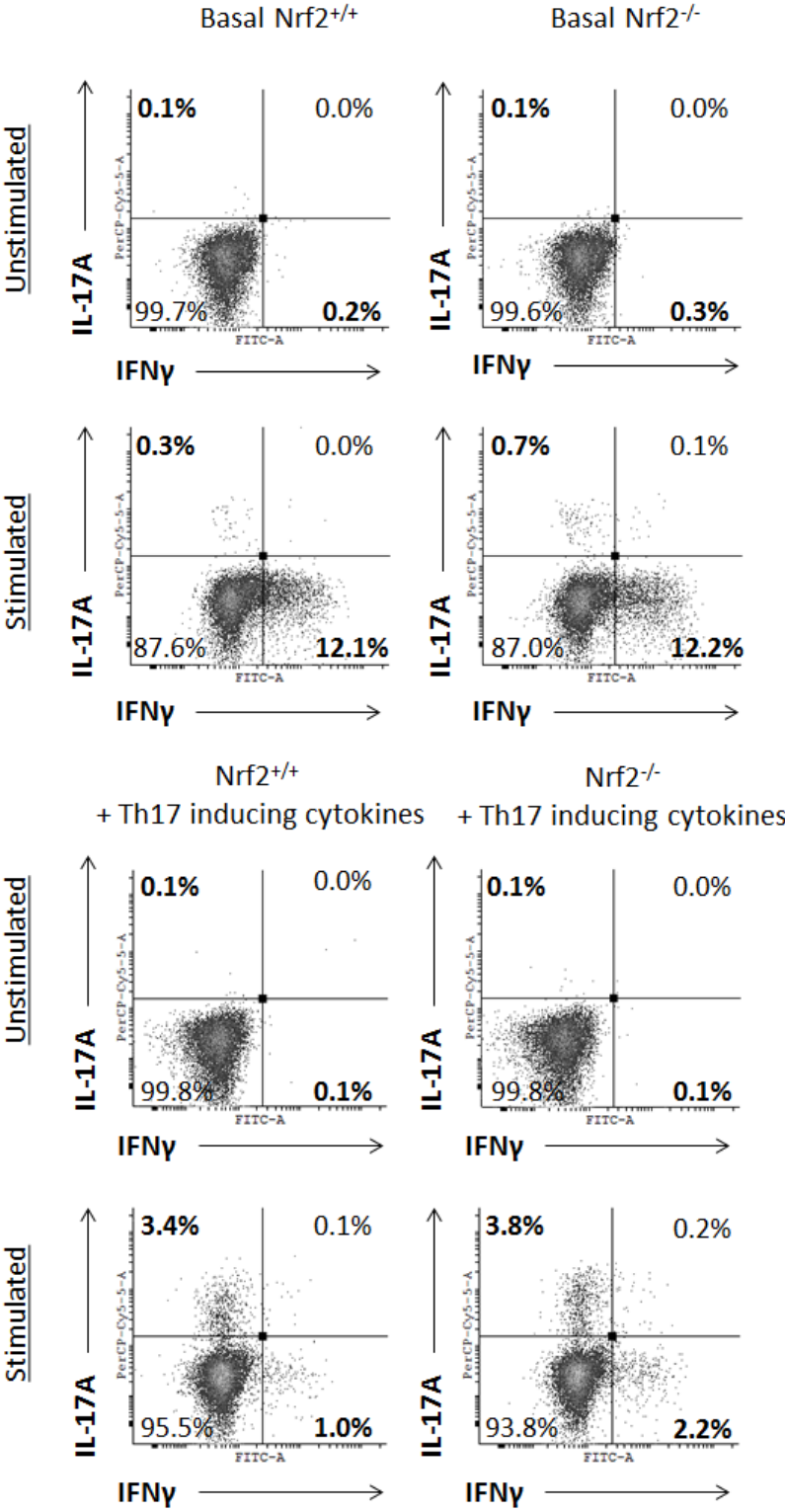
4.2.11 Loss of Nrf2 results in enhanced differentiation of CD4⁺IL-17⁺ splenic effector T cells

In light of our previous finding that Nrf2 deficient effector CD4 T cells produced elevated levels of IL-17, we speculated that loss of Nrf2 may predispose CD4 T cells to differentiate into effector Th17 cells, under Th17 lineage polarisation conditions (Mangan et al., 2006; Veldhoen et al., 2006). In order to explore this, purified naïve Nrf2^{+/+} and Nrf2^{-/-} CD4 T cells were treated with or without a Th17 inducing cytokine cocktail consisting of IL-6, TGFβ, IL-23 and IL-1β to promote Th17 polarisation (Mangan et al., 2006; Veldhoen et al., 2006). Cells were stimulated with immobilised anti-CD3 and anti-CD28 as before and cultured for 6 days to enable differentiation into effector cells. Day 6 Nrf2^{+/+} and Nrf2^{-/-} CD4 T cells in the

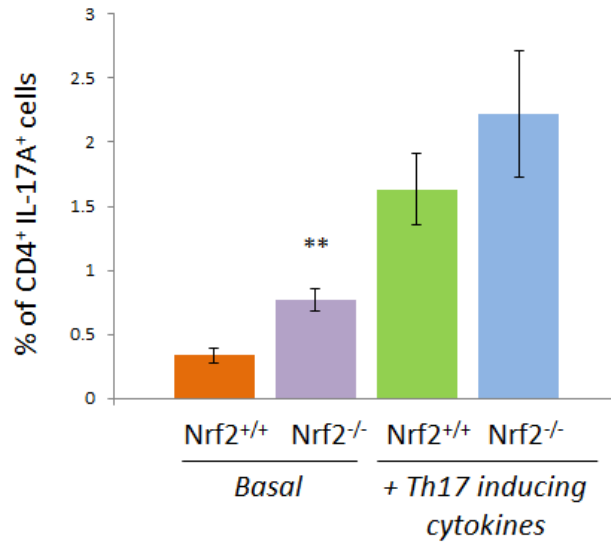
presence or absence of Th17 polarising cytokines were re-stimulated with or without PMA, ionomycin and immobilised anti-CD3 for 5 hours. Cells were then stained with cell surface CD4^{PE} and subsequently assessed for intracellular cytokine production of IL-17A^{PerCp-Cy5.5} and IFN γ ^{FITC} by flow cytometry. Cells were gated on the high CD4 population and simultaneous assessment of the percentage CD4 IL-17A and or IFN γ producing cells were determined by dot plot as represented in **Figure 4.11A**. Effector CD4⁺IL-17⁺ and CD4⁺IFN γ ⁺ T cells were shown in the upper left and lower quadrant sections within the dot plots, respectively (**Figure 4.11A**). Percentages of CD4⁺IL-17⁺ and CD4⁺IFN γ ⁺ T cells present in the stimulated effector T cells were subtracted from their respective unstimulated control and quantified in **Figure 4.11B**. Results revealed that basal Nrf2 deficient T cells exhibited significantly increased percentages of CD4⁺IL-17⁺ T cells in comparison to their wild type counterpart (**Figure 4.11Bi**, Nrf2^{+/+} 0.3 \pm 0.1% versus Nrf2^{-/-} 0.8 \pm 0.2%, p = 0.002). Under Th17 polarising conditions, both Nrf2^{+/+} and Nrf2^{-/-} splenic effector CD4 T cells showed increased numbers of CD4⁺IL-17⁺ T cells in comparison to their respective basal controls (**Figure 4.11Bi**, Nrf2^{+/+} 0.3 \pm 0.1% versus Nrf2^{+/+} + Th17 inducing cytokines 1.6 \pm 0.3%, p = 0.001; Nrf2^{-/-} 0.8 \pm 0.2% versus Nrf2^{-/-} + Th17 inducing cytokines 2.2 \pm 0.5%, p = 0.009). However, there were no significant differences in CD4⁺IL-17⁺ T cells numbers between the Th17 polarised Nrf2^{+/+} and Nrf2^{-/-} splenic effector CD4 T cells (**Figure 4.11Bi**). In contrast to our previous finding, there was no significant changes in the percentage of CD4⁺IFN γ ⁺ T cells under this type of re-stimulation between the Nrf2^{+/+} and Nrf2^{-/-} mice. It has been established that IFN γ inhibits the development of Th17 cells and thus Th17 cells secrete minimal levels of this Th1 specific cytokine (Harrington et al., 2005). Our results indicate that under Th17 polarising conditions, a significant decrease in CD4⁺IFN γ ⁺ T cells was observed in both Nrf2^{+/+} and Nrf2^{-/-} splenic effector CD4 T cells in comparison to their respective basal control (**Figure 4.11Bii**, Nrf2^{+/+} 17.8 \pm 4.2% versus Nrf2^{+/+} + Th17 inducing cytokines 4.2 \pm 1.5%, p = 0.01; Nrf2^{-/-} 21.3 \pm 5.8% versus Nrf2^{-/-} + Th17 inducing cytokines 5.2 \pm 1.4%, p = 0.02). However no significant differences were present between the Th17-polarised Nrf2^{+/+} and Nrf2^{-/-} splenic effector CD4 T cells. Taken together, this suggests that loss of Nrf2 enhances

the proportion of CD4⁺IL-17⁺ T cells under normal conditions Nrf2 appears to play under Th17 polarising conditions.

A



Bi



Bii

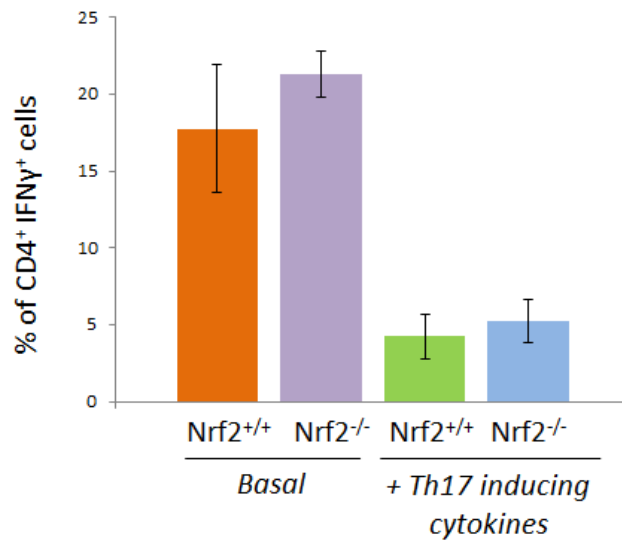


Figure 4.11 Splenic Nrf2^{-/-} effector T_H cells exhibit increased levels of CD4 IL-17 producing cells. CD4 purified Nrf2^{+/+} and Nrf2^{-/-} T cells were cultured in the presence or absence of Th17 inducing cytokine cocktail (TGFβ, IL-1β, IL-6 and IL-23) for 6 days. Cells were re-stimulated with plate bound CD3 antibody, PMA and ionomycin for 5 hours and assessed for intracellular cytokine production of IFNγ and IL-17A. Cells left unstimulated were used as background control. **A.** Representative dot plot of Nrf2^{+/+} (left) and Nrf2^{-/-} (right) effector T cells expressing IFNγ and IL-17A under basal (upper panel) of Th17 polarising (lower panel) conditions. Cells were gated on CD4⁺ high populations. Quantification of the average percentage of **Bi.** CD4 IL17A and **Bii** CD4 IFNγ cells in basal Nrf2^{+/+} (orange) and Nrf2^{-/-} (purple), and Th17 polarised Nrf2^{+/+} (green) and Nrf2^{-/-} (blue) effector T cells. Percentages were subtracted from unstimulated control. Data are presented as average percentage of T cells ± S.E.M. Statistical significance was assessed using unpaired Student's *t* test. Data are derived from five independent experiments (**, *p* < 0.01).

4.2.12 Loss of Nrf2 results in enhanced CD4 effector Th17 specific cytokine production under basal and Th17 polarising conditions.

Although loss of Nrf2 does not appear to enhance CD4⁺IL-17⁺ or CD4⁺IFN γ ⁺ T cells under Th17 polarising conditions, we cannot rule out that per cell these populations could potentially be producing more cytokine in comparison to the wild type control. To examine this possibility, day 6 Th17-polarised or basal Nrf2^{+/+} and Nrf2^{-/-} splenic effector CD4 T cells were re-stimulated with plate bound anti-CD3 and anti-CD28 overnight and cytokine production was assessed by ELISA. In line with our previous finding, Nrf2^{-/-} splenic effector CD4 T cells displayed elevated levels of IL-17A production in comparison to wild type control (**Figure 4.12A**, Nrf2^{+/+} not detected 0.0 ± 0.0 pg/ml versus Nrf2^{-/-} 1383.9 ± 146.0 pg/ml, $p = 0.004$). Under Th17 polarising conditions, our results clearly demonstrate that polarised Nrf2^{-/-} splenic effector CD4 T cells secreted increased levels of IL-17A in comparison to the wild type control (**Figure 4.12A**, Nrf2^{+/+} + Th17 cytokines 2655.9 ± 212.4 pg/ml versus Nrf2^{-/-} + Th17 cytokines 6239.7 ± 490.7 pg/ml, $p = 0.0003$), thus supporting the notion that the Nrf2 deficient effector cells were secreting more cytokine on a per cell basis. In line with **Figure 4.10**, basal Nrf2 deficient effector CD4 T cells exhibited increased levels of IFN γ production in comparison to Nrf2^{+/+} effector T cells (**Figure 4.12B**, Nrf2^{+/+} 87704.3 ± 2930.0 pg/ml versus Nrf2^{-/-} 113150.9 ± 7724.7 pg/ml, $p = 0.04$). Under Th17 polarising conditions, a significant decrease in IFN γ production was observed in both Nrf2^{+/+} and Nrf2^{-/-} splenic effector CD4 T cells with respect to their basal control (**Figure 4.12B**, Nrf2^{+/+} 87704.3 ± 2930.0 pg/ml versus Nrf2^{+/+} + Th17 inducing cytokines 9956.6 ± 457.3 pg/ml, $p = 0.001$; Nrf2^{-/-} 113150.9 ± 7724.7 pg/ml versus Nrf2^{-/-} + Th17 inducing cytokines 20041.3 ± 4027.1 pg/ml, $p = 0.0004$). An increase in IFN γ production was observed in the Th17-polarised Nrf2 deficient CD4 T cells in comparison to their wild type counterpart; however, this was not statistically significant ($p = 0.07$). We next assessed the level of IL-22 secretion between re-stimulated basal and Th17-polarised Nrf2^{+/+} and Nrf2^{-/-} splenic effector CD4 T cells. As IL-22 is a key cytokine produced by the distinctive Th17 effector subset (Zhu and Qian, 2012), it would be reasonable to assume that Nrf2 deficient T cells would produce enhanced IL-22 levels, as a consequence of their

increased IL-17 production. Results revealed that basal Nrf2^{+/+} and Nrf2^{-/-} splenic CD4 T cells secreted very low levels of IL-22. Nonetheless, basal Nrf2 deficient effector CD4 T cells still produced significantly more IL-22 than Nrf2^{+/+} splenic CD4 T cells (**Figure 4.12C**, Nrf2^{+/+} 1.81 ± 3.1 pg/ml versus Nrf2^{-/-} 23.4 ± 5.3 pg/ml, $p = 0.004$). Under Th17 polarising conditions, both Nrf2^{+/+} and Nrf2^{-/-} splenic CD4 T cells exhibited augmented IL-22 levels with respect to their basal control (**Figure 4.12C**, Nrf2^{+/+} 1.81 ± 3.1 pg/ml versus Nrf2^{+/+} + Th17 inducing cytokines 47.1 ± 8.5 pg/ml, $p = 0.001$; Nrf2^{-/-} 23.4 ± 5.3 pg/ml versus Nrf2^{-/-} + Th17 inducing cytokines 154.5 ± 6.6 pg/ml, $p < 0.0001$). Furthermore, polarised Nrf2 deficient T cells manifested enhanced IL-22 production in comparison to their wild type counterpart (**Figure 4.12C**, Nrf2^{+/+} + Th17 inducing cytokines 47.1 ± 8.5 versus Nrf2^{-/-} + Th17 inducing cytokines 154.5 ± 6.6 pg/ml, $p < 0.0001$). We have established that loss of Nrf2 results in enhanced Th17 specific cytokine production of IL-17A and IL-22 under polarising conditions. However, it is unknown whether loss of Nrf2 has an impact on effector Th2 IL-5 production under Th17 polarising conditions. Results depicted a significant decrease in IL-5 production was observed in both Th17-polarised Nrf2^{+/+} and Nrf2^{-/-} splenic effector CD4 T cells with respect to their basal control (**Figure 4.12D**, Nrf2^{+/+} 869.5 ± 62.0 pg/ml versus Nrf2^{+/+} + Th17 inducing cytokines 7.3 ± 1.4 pg/ml, $p = 0.002$; Nrf2^{-/-} 741.9 ± 22.1 pg/ml versus Nrf2^{-/-} + Th17 inducing cytokines 38.6 ± 3.0 pg/ml, $p = 0.0003$). Intriguingly, results revealed that under polarising conditions, Nrf2 deficient effector T cells secreted higher levels of IL-5 in comparison to their wild type counterpart as shown in **Figure 4.12D** (Nrf2^{+/+} + Th17 inducing cytokines 7.3 ± 1.4 pg/ml versus Nrf2^{-/-} + Th17 inducing cytokines 38.6 ± 3.0 pg/ml, $p < 0.0001$). Taken together, these results suggest the loss Nrf2 results in the enhanced predisposition of Th17 differentiation in CD4 T cells under basal and Th17 polarising conditions.

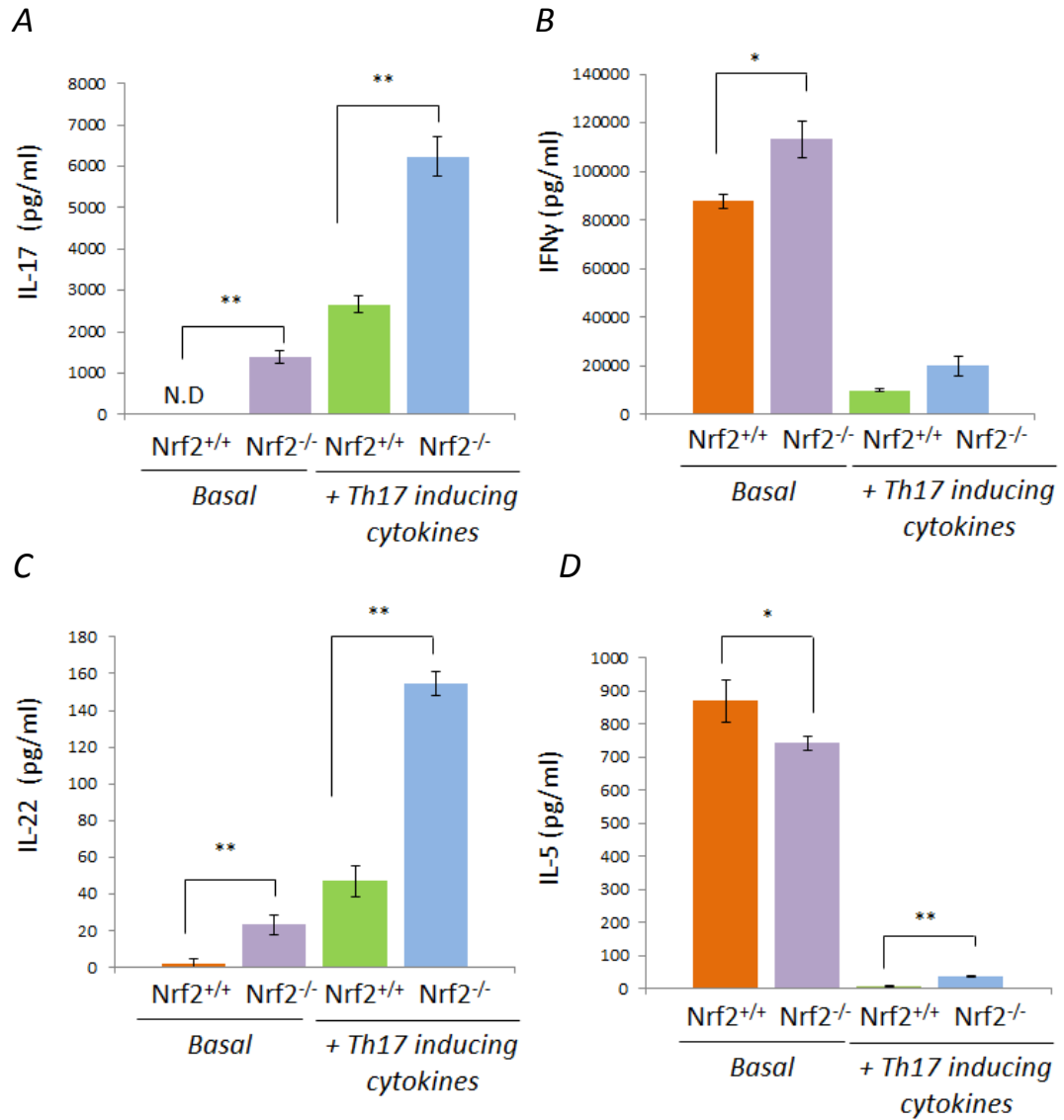


Figure 4.12 Nrf2 regulates CD4 effector T cell signature cytokine production. CD4 purified Nrf2^{+/+} and Nrf2^{-/-} T cells were cultured in the presence or absence of Th17 inducing cytokine cocktail (TGF β , IL-1 β , IL-6 and IL-23) for 6 days. Cells were re-stimulated with immobilised anti-CD3 and anti-CD28 antibodies for 24 h. Levels of **A.** IL-17A, **B.** IFN γ , **C.** IL-22 and **D.** IL-5 in supernatants were measured by ELISA. Data are presented as average pg/ml \pm S.D. Data is representative of three independent experiments. Statistical significance was assessed using unpaired Student's *t* test. (*, *p* < 0.05; **, *p* < 0.01).

4.3 DISCUSSION

The transcription factor Nrf2 plays a pivotal role in the regulation of antioxidant and cytoprotective genes involved in the counteraction of oxidative insults. Therefore, it is indispensable for the maintenance of cellular redox homeostasis (Copples et al., 2010). Evidence has emerged on the anti-inflammatory effects of Nrf2 in a variety of oxidative murine disease models including EAE (MS), sepsis, emphysema, asthma and brain neuroinflammation (Rangasamy et al., 2005; Thimmulappa et al., 2006b; Innamorato et al., 2008; Sussan et al., 2009; Johnson et al., 2010). Furthermore, Nrf2 has been shown to regulate innate immune responses with respect to macrophage and DC immune function (Williams et al., 2008; Reddy et al., 2009a; Kong et al., 2010; Rangasamy et al., 2010; Kong et al., 2011; Aw Yeang et al., 2012). However, little is known about the impact of this transcription factor on the maintenance of adaptive immune responses, in particular T cells. The present chapter investigated the role of Nrf2 in T cell development and immune function. Our results demonstrated that Nrf2 was not a critical driving force of T cell development within the thymus and peripheral secondary lymphoid organs. However, it did play an important role in the regulation of naïve T cell intracellular ROS levels, T cell activation and proliferation. Furthermore, our results established a role of Nrf2 in the maintenance of CD4 T cell differentiation, particularly in Th17 development. This latter finding is of huge relevance given the emerging role of the newly identified Th17 cells in the pathogenesis of autoimmune diseases, thus merits intense investigations.

Reflecting on the role of Nrf2 in T cell development within the thymus, although we determined that Nrf2 plays a minor role in the overall thymic T cell proportions, we have not fully assessed the impact of Nrf2 on the various stages of thymocyte differentiation within the DN population. As immature thymocytes transverse through the thymic cortex, they proceed through DN1 – DN4 stages of differentiation as typified by CD44 and CD25 expression; ensuring the development of competent TCR and progression to the DP phase (Germain, 2002). It is feasible that Nrf2 may modulate the degree of differentiation from DN1 through to DN4 maturation stages. Additional staining of CD44 and CD25 on the gated DN cells

would resolve this matter. Furthermore, SP CD4 and CD8 T cells undergo various stages of maturation within the thymic medulla as characterised by differential CD69 and CD62L expression (Rosen et al., 2003). Our results demonstrated that there were no changes in the expression of these markers in the Nrf2 deficient thymocytes. Retrospectively, it would have been more informative to examine CD69 and CD62L simultaneously in the mature SP populations so as to achieve a finer resolution of the maturation stages within the thymic medulla. Loss of Nrf2 had no significant impact on the proportion of nTregs within the thymus with respect to CD25 expression. However, it would have been useful to examine the levels of FoxP3 expression in this population (Gavin et al., 2007). The expression of this transcription factor is necessary not only for their development but for their immunosuppressive function (Gavin et al., 2007), the latter of which could also have been examined. It has been proposed that another SP CD4- derived T cell population which are devoid of FoxP3 expression known as natural Th17 cells (nTh17), develop in a somewhat similar fashion to nTregs within the thymus (Kim et al., 2011; Zuniga et al., 2013). We observed that Nrf2 modulated Th17 differentiation in the periphery and although the development pathways between adaptive Th17 cells and nTh17 cells are distinct from one another (Kim et al., 2011), it would still be interesting to explore the role of Nrf2 in this relatively undiscovered thymic-derived T cell population.

We next deciphered whether loss of Nrf2 impacted on the composition of CD4 and CD8 T cell populations within peripheral lymphoid organs. Our results clearly demonstrated that no differences in CD4 and CD8 T cells ratios were exhibited between Nrf2^{+/+} and Nrf2^{-/-} mice within the spleen or lymph node. These results are in line with recently published data (Rockwell et al., 2012; Wu et al., 2013), wherein no overt splenic T cell phenotypic changes were seen in the Nrf2 deficient mouse. Loss of Nrf2 results in enhanced basal ROS levels in a variety of cell types including hepatocytes (Beyer et al., 2008), macrophages (Thimmulappa et al., 2006b) and DCs (Aw Yeang et al., 2012); however, it has yet to be shown in peripheral T cells. Our results revealed that Nrf2 deficient naive T cells exhibited enhanced basal ROS levels in relation to their wild type control. Nrf2^{-/-} naïve T cells did not display

significantly enhanced ROS levels in the presence of H_2O_2 in comparison to their baseline control, as was observed for the wild type mouse. This suggests that naïve Nrf2 deficient T cells' heightened basal ROS levels were already saturated hence added stress had no real impact on their overall ROS status. A previous study has shown that increased ROS levels during T cell development impaired the composition of CD4 and CD8 T populations in the spleen (Case et al., 2011). As we found no differences in T cell populations within the periphery between both genotypes, one would assume that no differences would be observed in basal Nrf2^{+/+} and Nrf2^{-/-} thymocyte ROS levels; nevertheless, this warrants investigation. Reactive oxygen species is required for competent T cell activation, proliferation and differentiation (Shatynski et al., 2012; Sena et al., 2013). As the naïve Nrf2 deficient T cells exhibited enhanced basal ROS levels, we postulated that this would impact on the basal activation status of the mature peripheral T cells, in terms of CD25, CD69, CD44 and CD62L expression. No differences in CD25 levels were observed between Nrf2^{+/+} and Nrf2^{-/-} peripheral T cells, which is in line with previously published data (Rockwell et al., 2012). Similar results were also observed for CD69 expression. In contrast, our results illustrated that Nrf2 deficient naïve T cells manifested signs of low level CD4 and CD8 T cell activation, with respect to enhanced CD44 and diminished CD62L expression in the lymph node. Augmented levels of CD44 were also noted in splenic Nrf2 deficient naïve CD4 T cells. This finding conflicts with that of Rockwell et al. where no differences in CD44 expression between Nrf2^{+/+} and Nrf2^{-/-} naïve splenic T cells were presented (Rockwell et al., 2012). Reasoning for this discrepancy could be attributable to the type of flow cytometric analysis performed. Rockwell et al. assessed CD44 expression within a CD4⁺CD25⁻ gated population, whereas our cells were gated on the CD4⁺ population only (Rockwell et al., 2012). In support of our finding, increased ROS levels in CD4 naïve T cells have been associated with down regulation in CD62L expression (Foster et al., 2013). It would be valuable to examine the precise impact of the naïve Nrf2 deficient T cell elevated ROS levels on subsequent effector T cell function. This could be achieved by pre-treating the Nrf2^{+/+} and Nrf2^{-/-} T cells with the antioxidants vitamin C and E which are known to

reduce ROS levels (Al-Huseini et al., 2013) and assessing effector T cell proliferation and effector cytokine production as below.

We speculated that the increased naïve T cell activation status exhibited in the Nrf2 deficient mouse would amount to enhanced naïve T cell proliferation upon antibody stimulation. However, no differences in splenic naïve T cell proliferative capacity were observed between Nrf2^{+/+} and Nrf2^{-/-} mice. A previous study has shown that loss of Nrf2 results in enhanced lymph node-derived naïve T cell proliferation in response to CD3 stimulation (Ma et al., 2006). Furthermore, they found a decrease in CD8 T cell numbers within the lymph nodes of the Nrf2 deficient mouse (Ma et al., 2006). The inconsistency between the results presented in this chapter and the aforementioned report is undoubtedly due to the age of mice used in both studies. Ma et al. utilised 6 month old mice which exhibited gross signs of phenotypic abnormalities and illness for their study; whereas 10-12 weeks old mice which were devoid of any overt illness were used in the presented study (Ma et al., 2006). Reasons for this discrepancy could also be based on the utilisation of Nrf2 deficient mice derived from distinctive inbred genetic backgrounds, 129SVJ and C57BL/6J in the case of Ma et al. (Ma et al., 2006) and the present study, respectively.

We next explored the role of Nrf2 in effector T cell immune function initially looking at their proliferative capacity in response to CD3 and CD28 antibodies. Our results demonstrated that loss of Nrf2 significantly enhanced effector T cell proliferation in response to increasing concentrations of antibody stimulation, a finding which has not been published to date. We reasoned that this increase in T cell proliferation would result in an increase in effector T cell immune function. Accordingly, we measured the level of effector CD4 and CD8 T cell intracellular IFN γ expression upon antibody re-stimulation through flow cytometric analysis. Our results indicated that there were no differences in the numbers of splenic effector CD8⁺IFN γ ⁺ T cells between Nrf2^{+/+} and Nrf2^{-/-} mice, suggesting that Nrf2 does not play a role in the regulation of this particular aspect of CD8 T cell immune function. It may be the case that both Nrf2^{+/+} and Nrf2^{-/-} CD8 T cells were secreting such high levels of IFN γ , that subtle changes (as was the case for CD4) could not be detected

by this method. However, other functional readouts of CD8 T cells should be considered to further clarify the role of Nrf2 in CTL immune function. Cytotoxic T lymphocyte cytolytic capacity could be evaluated using the chromium release assay (Janas et al., 2005). Furthermore, expression of CTL-released cytolytic molecules such as perforin and granzyme B could also be measured (Janas et al., 2005) by flow cytometry. In contrast to our effector CD8 T cell finding, Nrf2 deficient effector T cells displayed enhanced numbers of CD4⁺IFN γ ⁺ T cells in comparison to their wild type counterpart. This has not been shown before through this methodology. However, these numbers were approximately 2 fold lower than what was observed for the CD8 T cells. As effector IFN γ production is a characteristic Th1 cytokine (Hsieh et al., 1993), this suggests that Nrf2 plays a role in the CD4 polarisation through the modulation of Th1 differentiation. We sought to confirm this finding by evaluating the absolute amounts of IFN γ produced by purified CD4 Nrf2^{+/+} and Nrf2^{-/-} re-stimulated effector T cells. Our results demonstrated a clear increase in IFN γ levels in the Nrf2 deficient effector T cells. In support of our finding, Rockwell et al also presented that loss of Nrf2 in re-stimulated effector T cells induced elevated IFN γ secretion (Rockwell et al., 2012). Moreover, a similar increase was observed in antibody stimulated Nrf2^{-/-} CD4 naïve T cells (Roman et al., 2010), thus reaffirming our previous conclusion that Nrf2 regulates CD4 T cell differentiation, particularly Th1 development. Loss of Nrf2 was shown to reduce TNF α secretion by re-stimulated effector T cells. The converse was previously shown in antibody stimulated naïve Nrf2 deficient T cells which exhibited enhanced TNF α production (Rockwell et al., 2012). Rockwell et al. concluded that Nrf2 had little effect on naïve T cell TNF α production, as no real changes were observed in the wild type or knockout mouse, in response to additional increasing concentrations of *tert*-butylhydroquinone (tBHQ), a known activator of Nrf2 (Rockwell et al., 2012). However, they did not measure its production in re-stimulated effector T cells. As this proinflammatory cytokine is produced by both Th1 and Th2 cells (Zhu et al., 2010), it is not an adequate CD4 effector T cell signature cytokine to measure in order to pinpoint the precise role of Nrf2 in CD4 T cell lineage differentiation. It is well recognised that cross-regulation exists between Th1 and Th2 cells, in which Th1 cells inhibit the differentiation and immune function of Th2 cells, with the

converse also true (Szabo et al., 2000; Usui et al., 2003; Usui et al., 2006; Gu et al., 2012). Therefore, we hypothesised that increased CD4 effector IFN γ production would be associated with a down-modulation of Th2 cytokine production such as IL-5. Our results confirmed that Nrf2 deficient effector T cells manifested reduced levels of IL-5 secretion upon re-stimulation. This was reflected in the Rockwell et al. study which also examined the nuclear binding activity of CD4 master transcription factors T-bet (Th1) and GATA-3 (Th2) in Nrf2 deficient re-stimulated effector T cells. Their results revealed an increase and decrease in T-bet and GATA-3 binding, respectively, providing a mechanistic basis behind the observed reciprocal Th1/Th2 cytokine production. This further emphasises a pivotal role for Nrf2 in the maintenance of Th1/Th2 differentiation (Rockwell et al., 2012). The importance of maintaining a Th1/Th2 balance is highlighted in allergic disease settings such as asthma and eczema; pathogenesis which are driven by a dominant Th2 phenotype (Holgate, 2012). The pivotal role of Nrf2 in the regulation of the Th1/Th2 equilibrium has been previously shown in a murine asthma model (Rangasamy et al., 2005). In this study, the authors found no significant differences in splenic CD4 naïve T cell production of IL-4 and IL-13 upon antibody stimulation between Nrf2^{+/+} and Nrf2^{-/-} mice. However, substantial induction of Th2 cytokine secretion was observed in OVA-sensitised re-stimulated splenic CD4 T cells in comparison to their wild type control, indicating that Nrf2 down-modulates Th2 differentiation within this oxidative context (Rangasamy et al., 2005). We next sought to investigate whether loss of Nrf2 had any impact on CD4 effector IL-17 production, the signature cytokine associated with the more recently identified CD4 Th17 subtype (Harrington et al., 2005; Mangan et al., 2006). Although six IL-17 cytokine isoforms have been identified from IL-17A-F, the well characterised IL-17A isoform has been regarded as critical for the induction of autoimmunity and thus was chosen as an ideal candidate for the measurement of IL-17 within this study (Gaffen, 2009). Loss of Nrf2 has been shown to exacerbate disease severity in a murine model of MS (Johnson et al., 2010). Therefore one would postulate that this may be attributable to Nrf2 deficient mediated augmentation of Th17 cell effector function. Results revealed that loss of Nrf2 increased IL-17A secretion in effector T cells, implicating a role for Nrf2 in Th17 development. This is of great relevance given the emerging

role of Th17 in anti-fungal, anti-bacterial immunity and its notorious role in the immunopathology of a variety of autoimmune disorders including SLE, MS and RA (Zhu and Qian, 2012). We further speculated that loss of Nrf2 results in a predisposition of CD4 T cells to develop into the Th17 lineage basally and under Th17 polarising conditions. In order to test the latter, Nrf2^{+/+} and Nrf2^{-/-} effector T cells cultured with or without Th17 inducing cytokines (TGFβ, IL-6, IL-23, IL-1β) (Mangan et al., 2006; Veldhoen et al., 2006) were assessed for intracellular IL-17A and IFNγ. In contrast to our previous finding, no differences in CD4⁺IFNγ⁺ T cells were observed between basal Nrf2^{+/+} and Nrf2^{-/-} effector T cells under this type of re-stimulation. Similar results were attained for the Th17-polarised Nrf2^{+/+} and Nrf2^{-/-} effector T cells. Results reaffirmed that loss of Nrf2 resulted in an increase in basal CD4⁺IL17A⁺ T cells in comparison to their wild type counterpart. Surprisingly, this increase in IL-17 producing cells was not observed within the Th17 induced Nrf2 deficient effector T cell populations, suggesting that Nrf2 may play a negligible role in Th17 development. However, we reasoned that although Th17 polarised Nrf2^{+/+} and Nrf2^{-/-} effector T cells had similar proportions of CD4⁺IL17A⁺ T cells, they were capable of secreting enhanced absolute amounts of IL-17 per cell basis. Our results confirmed by ELISA that Nrf2^{-/-} effector T cells secreted augmented levels of IL-17A basally and under Th17 polarising conditions. Furthermore, basal Nrf2^{-/-} effector T cells exhibited enhanced IFNγ. It is recognised that a reciprocal relationship exists between Th1 and Th17 cells (Nurieva et al., 2007; El-Behi et al., 2011), such that IFNγ inhibits Th17 development (Harrington et al., 2005). Therefore it is surprising that Nrf2^{-/-} CD4 effector T cells produce enhanced levels of both IL-17A and IFNγ. It has been shown that the Nrf2 regulated gene HO-1 controls T cell-mediated IFNγ production in response to antibody stimulation (Kapturczak et al., 2004) and regulates the percentage of CD4⁺IL-17⁺ T cells in murine EAE model (Tzima et al., 2009). Loss of Nrf2 results in a decrease in HO-1 expression in T cells (Rockwell et al., 2012); therefore, the results presented in the Nrf2 deficient mouse may be attributable to loss of HO-1 expression rather than Nrf2 deficiency itself. Pharmacological inhibition of HO-1 expression using Sn-protoporphyrin (Zhong et al., 2010) in the wild type CD4 T cells would allow us to delineate the role of HO-1 in CD4 T cell IL-17A and IFNγ production. Nrf2 deficient basal and TH17-induced

effector T cells secreted higher levels of IL-22, another Th17 signature cytokine (Zhu and Qian, 2012), in relation to their wild type control. These results further emphasise the role of Nrf2 in the regulation of Th17 development. However, this increase in IL-22 may also point towards an increase in the recently identified Th22 cells. Effector Th22 cells secrete IL-22 independently of IL-17 and are emerging as a key player in the pathogenesis of an array of atopic allergic disorders such as eczema and psoriasis (Eyerich et al., 2009). Therefore it would be worthwhile to perform an intracellular stain for IL-22 and IL-17A simultaneously, to compare the number of CD4⁺IL17⁻IL-22⁺ cells between Nrf2^{+/+} and Nrf2^{-/-} mice, under basal and polarised conditions. A very recent study investigating the role of Nrf2 in heart graft rejection, illustrated enhanced mRNA IL-17 levels in the grafts derived from Nrf2 deficient recipient mice in comparison to wild type control (Wu et al., 2013). Oleanoic acid and its triterpenoid derivative CDDO-trifluoroethyl-amide (CDDO-TFEA) which are known to activate Nrf2 reduce disease severity in murine EAE model which is associated with decreased IL-17 levels in the sera and spinal cord of CNS affected mice (Pareek et al., 2011; Martin et al., 2012). Pre-treatment with another synthetic triterpenoid CDDO-Me limits IL-17, IL-6 and IL-23 production in murine plasma upon *in vivo* challenge with LPS (Auletta et al., 2010). Taken together, these further support the role of Nrf2 in the modulation of Th17 development. Interestingly, Th17-polarised Nrf2^{-/-} CD4 effector T cells secreted enhanced IL-5 levels in comparison to their wild type counterpart, which is opposite to what we observed in the basal effector T cells. This is surprising given the inverse relationship between Th2 and Th17 cells (van Hamburg et al., 2008; Laurence et al., 2012). Murine STAT6^{-/-} mice (STAT6 is a known Th2 regulator (Zhu et al., 2010)) displayed enhanced Th1 and Th17 cells in experimental crescentic glomerulonephritis, which was associated with a significant decrease in IL-5 production (Summers et al., 2011). Furthermore, neutralisation of IL-17 during murine corneal graft transplantation was shown to increase IL-5 levels in the spleen of recipient mice further implying reciprocal Th17/Th2 cytokine production (Chen et al., 2011).

A strikingly obvious limitation to the Th17 differentiation protocol utilised in this present chapter is the low percentage of effector CD4⁺IL-17⁺ T cells yielded in comparison to other published data (Mangan et al., 2006; Veldhoen et al., 2006; Veldhoen et al., 2009). In fact the literature itself is extremely varied in the numbers of effector CD4⁺IL-17⁺ T cells produced (ranging from 5-60%), owing to diverse range of protocols used to differentiate the Th17 cells (Mangan et al., 2006; Veldhoen et al., 2006; Veldhoen et al., 2009; Zhi et al., 2012). These include the incorporation of blocking antibodies for IL-2, IFN γ and IL-4 (Mangan et al., 2006; Zhi et al., 2012), increasing the concentrations of IL-6 (Zhi et al., 2012) and using of the aromatic amino acid-rich Iscove's Modified Dulbecco's Medium (IMDM) for culture rather than RPMI (Veldhoen et al., 2009). It is therefore imperative to optimise this Th17 differentiation method further, to improve yield for subsequent functional studies that require large numbers of cells.

It would have been interesting to evaluate the levels of the master transcription factor ROR γ t (Ivanov et al., 2006; Burgler et al., 2010) between Nrf2^{+/+} and Nrf2^{-/-} mice through flow cytometric analysis, to further reaffirm our findings. Moreover, STAT3 is obligatory for Th17 development whilst STAT1 (Th1 inducing STAT) and STAT5/STAT6 (Th2 inducing STATs) have been known to inhibit Th17 development, thus it would be worth examining their protein expression between the two genotypes to uncover a mechanistic basis for our presented findings (Harrington et al., 2005; Laurence et al., 2007; Mathur et al., 2007). Transcriptional activity of AHR has also been shown to promote Th17 polarisation (Veldhoen et al., 2008; Veldhoen et al., 2009). Inhibition of this receptor or supplementation of AHR ligands in *in vitro* cultures results in impaired or optimised Th17 differentiation, respectively (Veldhoen et al., 2009). It is recognised that an integral relationship lies between Nrf2 and AHR. The nuclear aryl hydrocarbon receptor is activated by xenobiotic ligands including 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), resulting in its nuclear translocation. Similarly to Nrf2 which binds to the ARE, AHR binds to xenobiotic response elements (XRE) present within the promoter regions of its regulated genes including phase I metabolising cytochrome p450 enzymes, resulting in their upregulation and induction of drug metabolism (Wakabayashi et

al., 2010). The dioxin TCDD induces the expression of phase II metabolising enzyme NQO1 which was dependent on both Nrf2 and AHR signalling (Ma et al., 2004). Moreover, rat exposure to cigarette smoke resulted in the enhancement of both Nrf2 and AHR expression within the brain (Khanna et al., 2013). In contrast, green tea extract epigallocatechin gallate has been shown to attenuate polychlorinated biphenyl (AHR ligand)-mediated inflammation in endothelial cells through the inhibition of the AHR pathway. This was associated with a downregulation of AHR-dependent cytochrome P450 1A1 expression, whilst enhancing the induction of Nrf2 related genes e.g. GST and NQO1 (Han et al., 2012). In keeping with the suggestion that cross regulation exists between Nrf2 and AHR, it would be reasonable to postulate that loss of Nrf2 could possibly result in an increase in AHR signalling thus promoting the development of Th17 cells exhibited in the Nrf2 deficient mice. Addressing the latter could be achieved by comparing differences in AHR nuclear protein levels in re-stimulated Nrf2^{+/+} and Nrf2^{-/-} effector T cells in the presence or absence of Th17 inducing cytokines. Furthermore, AHR activation results in the upregulation of downstream cytochrome P450 enzymes CYP1A1 and CYP1A2 gene expression (Stockinger et al., 2011). The mRNA levels of CYP1A1 and CYP1A2 could therefore be examined via RT-PCR within these T cells. The glycolytic metabolic activity of Th17 cells is highly dependent on the transcriptional activity of Hypoxia-inducible factor-1 α (HIF-1 α) which further promotes Th17 generation (MacIver et al., 2013). Similarly to Nrf2 in its role in the regulating cellular redox, HIF-1 α is the key player responsible for the maintenance of cellular oxygen homeostasis (Semenza, 2003). It upregulates genes involved in cell proliferation, cell survival, angiogenesis and glucose metabolism in response to cellular stress induced by hypoxia (Semenza, 2003). A study showed that activation of HIF-1 α by dimethyloxaloylglycine attenuated Nrf2-dependent production of IL-8 in endothelial cells, a result that was reversed upon Nrf2 overexpression (Loboda et al., 2009). Hypoxia-inducible factor-1 α is activated via dimerisation with the AHR nuclear translocator (ARNT), which in turn regulates that activity of AHR (Beischlag et al., 2008). It has been recently demonstrated that ARNT deficiency in CD4 T cells results in reduction in Th17 differentiation (Kim et al., 2013). It would be interesting to examine the nuclear binding activity of HIF-1 and expression of ARNT between the

two Nrf2^{+/+} and Nrf2^{-/-} mice to delineate a potential mechanism to the enhanced Th17 phenotype exhibited in the Nrf2 deficient mouse.

Although we have established a role for Nrf2 in Th17 development *in vitro*, it is important to investigate this further in an *in vivo* murine model. The commonly utilised T cell-mediated hepatitis murine model, in which the T cell mitogen Con-A is injected intravenously into the mouse, has been shown to induce Th17 development (Li et al., 2012a). Moreover, Con-A induced T cell hepatitis has been previously implemented in the Nrf2 deficient mouse (Osburn et al., 2008). Taken together, this provides us with a sound rationale for the exploitation of this model in exploring the role of Nrf2 in Th17 development *in vivo*. Nrf2 deficient predisposition to induction of Th17 lineage could be assessed by examining the sera levels of IL-6, TGFβ, IL-1β, IL-23 (all of which are important for induction of Th17 cells) IL-17A, IL-22 (Th17 signature cytokines) IFNγ (Th1) and IL-5 (Th2) in the Con-A treated Nrf2^{+/+} and Nrf2^{-/-} mice. The aforementioned cytokines could also be examined in addition to percentages of CD4⁺IL17⁺ and CD4⁺IFNγ⁺ T cells in antibody-stimulated splenocytes derived from Con-A treated mice.

In conclusion, the presented findings reveal a novel role of Nrf2 in the regulation of Th17 development. Effector Th17 cells has been implicated in the pathogenesis of a variety of autoimmune diseases such as MS, SLE, RA and colitis, therefore targeting Nrf2 in these disease settings may potentially reduce the disease severity. Although this chapter has pinpointed a relevant immune function of Nrf2 in the modulation of Th17 polarisation, the precise role of Nrf2 in Th17 development remains to be defined and requires extensive research.

CHAPTER FIVE

INTER-INDIVIDUAL VARIATION IN THE CDDO-ME INDUCED NRF2 RESPONSE IN HUMAN T CELLS

5.1 INTRODUCTION

Adverse drug reactions (ADRs) are a significant health problem contributing to 6.5% of hospital admissions and resulting in post market drug attrition (Pirmohamed et al., 2004). Adverse drug reactions fall into 2 main categories, Type A and type B reactions. Type A (on-target) reactions are most common, accounting for approximately 80% of ADRs. These predictable reactions are concentration-dependent, related to the pharmacological action of the drug and are associated with low mortality; an example of which being digoxin toxicity (Edwards and Aronson, 2000). In contrast, the less common Type B (off-target) idiosyncratic ADRs are unpredictable, concentration-independent and are associated with high mortality (Edwards and Aronson, 2000). Idiosyncratic ADRs are often immune-mediated, examples of which include methotrexate-induced and penicillin-induced anaphylaxis (Uetrecht, 2005; Oulego-Eroz et al., 2010); hydralazine-induced lupus erythematosus (Antonov et al., 2004), SMX-induced eczema (Castrejon et al., 2010), carbamazepine-induced agranulocytosis (Zaccara et al., 2007) and methyl-dopa-induced hepatitis (Slim et al., 2010). Understanding the mechanisms behind idiosyncratic ADRs are extremely complicated due to human individual variation in responses to the therapeutic drug concentration, ranging from mild to severe reactions (Edwards and Aronson, 2000). Susceptibility to these unpredictable ADRs are highly influenced by various individual-specific factors including age, gender, lifestyle, disease status, drug-drug interactions and importantly genetic makeup (Severino and Del Zompo, 2004). Drug metabolism occurs primarily in the liver which entails the biotransformation of non-polar, lipophilic parent drugs into polar, water-soluble compounds; ultimately facilitating their safe removal from the body (Park et al., 2005a). This is achieved through the activity of phase I (e.g. cytochrome P450 enzymes) and phase II (e.g. GST) drug-metabolising enzymes; involved in xenobiotic functionalization and detoxification, respectively (Park et al., 2005a). However, the process of drug metabolism results in the generation of ROS and highly reactive drug metabolites which induce oxidative stress. This can potentially lead to cell death and tissue injury as highlighted in drug-induced liver injury (DILI) (Park et al., 2005a). A key component involved in the nullification of xenobiotic-

mediated oxidative stress is the adaptive Nrf2 pathway. In response to electrophilic or oxidative insult, Nrf2 drives the induction of a battery of cytoprotective and antioxidant defence systems resulting in the maintenance of cellular redox homeostasis and prevention of cellular damage (Copple et al., 2010). Studies using the Nrf2 deficient mice have highlighted the crucial role of Nrf2 in protection against xenobiotic-induced organ injury including acetaminophen-induced hepatotoxicity (Enomoto et al., 2001; Goldring et al., 2004), cisplatin-induced nephrotoxicity (Aleksunes et al., 2010) and bleomycin-induced pulmonary fibrosis (Cho et al., 2004; Kikuchi et al., 2010). However, Nrf2-mediated protection against xenobiotic-induced toxicity has yet to be identified in primary human cells. It is established that dietary administration of coffee constituent 5-O-caffeoylquinic acid and sulforaphane-rich broccoli sprout extract activates the Nrf2 adaptive system in human PBMCs, through the induction of Nrf2-regulated gene expression of NQO1 and HO-1 (Boettler et al., 2011b; Harvey et al., 2011). Moreover, the Nrf2 inducing triterpenoids CDDO-Im and CDDO-Me have been shown to enhance nuclear accumulation of Nrf2 and downstream gene expression in human PBMCs (Thimmulappa et al., 2007). It has been suggested that the adaptive Nrf2 defence system itself is varied between individuals (Marzec et al., 2007; Boettler et al., 2012). Previous reports have indicated that potential variation in human Nrf2 adaptive response exists, in response to Nrf2 inducers such as CDDO-Im and the coffee constituent 5-O-caffeoylquinic acid in human PBMCs. (Thimmulappa et al., 2007; Boettler et al., 2011b). However, the aforementioned studies did not exclusively examine inter-individual variation in the Nrf2 response and thus merits investigation. Could susceptibility to idiosyncratic ADRs in humans, therefore, be correlated with variation in their Nrf2 adaptive response to xenobiotic stress? To begin addressing this complex question, it is important to firstly demonstrate the existence of inter-individual variation in the induction of the Nrf2 adaptive system in response to a known Nrf2 probe, within a suitable human *ex vivo* experimental model. This should consist of an amenable source of easily obtained cells such as blood-derived immune cells, which are also capable of exhibiting Nrf2-mediated responses. The hypothesis of the work described in this chapter is that individuals vary in their adaptive Nrf2 response to a known Nrf2 inducer. The aim of this

chapter was to optimise a method in which to measure and quantify differences in Nrf2 protein and downstream NQO-1 gene expression in homogenous human T cells, derived from a small group of healthy subjects, in response to increased concentrations of a known Nrf2 probe. As CDDO-Me is known to induce Nrf2 responses in human immune cells (Thimmulappa et al., 2007), it was chosen as a candidate chemical Nrf2 inducer within our human T cell experimental model.

5.2 RESULTS

5.2.1 Nrf2 was undetectable in whole cell lysates of human T cells

To test the hypothesis that individual's vary in their Nrf2 adaptive response to an Nrf2 probe, we first sought to establish an appropriate human *ex vivo* experimental system, which utilised relevant accessible adaptive immune cells for the detection of basal and induced Nrf2 protein expression. In order to achieve the latter, human PBMCs were isolated from heparinised venous whole blood through density gradient centrifugation. The PBMCs were subsequently stimulated with the human T-cell mitogen, phytohaemagglutinin (PHA) and co-cultured with IL-2, ensuring the expansion of highly purified CD3⁺ T cell blasts (**Figure 5.1A**, CD3⁺ T cells: 98 %); large numbers of which were required for the biochemical nature of the presented pilot study. It is recognised that CDDO-Me induces Nrf2 expression in a variety of human cell types including PBMCs and neutrophils (Thimmulappa et al., 2007). Therefore, it was chosen as an appropriate candidate for Nrf2 induction in the human day 5 PHA-stimulated T cells and whole cell lysates (WCL) were prepared. Human T cells were treated with increasing concentrations of CDDO-Me (0-100nM) for 2 hours. It has been established that the proteasomal inhibitor MG132 induces Nrf2 expression in HepG2 human hepatoma cells WCL and thus was used as a positive control (Jeong et al., 2005). Whole cell expression of Nrf2 was assessed by western blot. Results revealed that under basal or CDDO-Me inducing conditions, Nrf2 was not detected in the WCL of human CD3⁺ T cell blasts (**Figure 5.1B**). The validity of this result was confirmed by substantial induction of Nrf2 expression exhibited in the MG132-treated HepG2 positive control. This suggests that Nrf2 protein content

is extremely low in human T cells; therefore it would be valuable to examine the content of Nrf2 in a smaller, more enriched location, namely the nucleus.

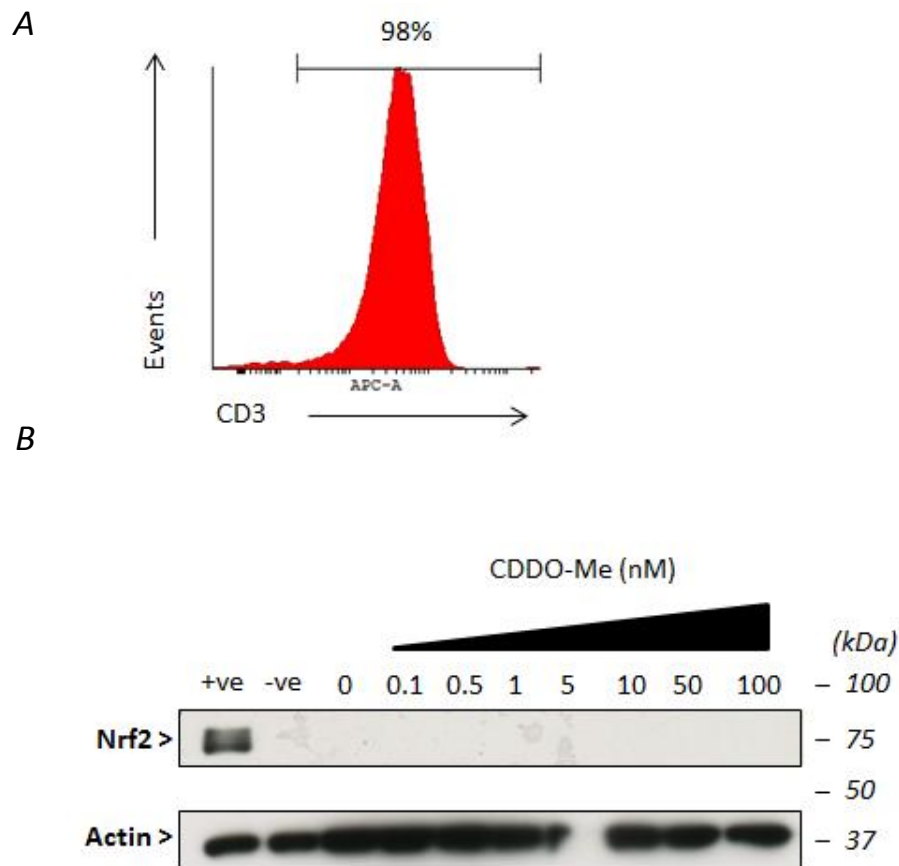


Figure 5.1 Nrf2 is not detected in the whole cell lysates of human T cells upon CDDO-Me induction. **A.** Day 5 Phytohaemagglutinin (PHA)-stimulated T cells derived from human isolated PBMCs were stained with CD3 APC and analysed by flow cytometry to assess T cell purity. Percentage of CD3+ T cells is indicated above the histogram marker. Position of the marker is based on the unlabelled control. **B.** Human PHA-stimulated T cells were treated with increasing concentrations of CDDO-Me (0-100nM in 0.1 % DMSO) for 2 h and whole-cell extracts were prepared. Cell lysates (20µg) were subjected to SDS-PAGE, and levels of Nrf2 and the loading control protein β -actin were determined by western blot analysis. HepG2 cells treated with or without proteasome inhibitor MG132 (10µM) for 2 h were used as a positive and negative control, respectively.

5.2.2 CDDO-Me-mediated induction of Nrf2 is detected in the nucleus of human T cells

Under basal conditions, Nrf2 is sequestered in the cytosol by the repressor protein Keap-1, which facilitates Nrf2 polyubiquitination and subsequent proteasomal degradation. Thus, a minimal cytoplasmic pool of Nrf2 is maintained as a result of its rapid degradation (Kobayashi et al., 2004; Katoh et al., 2005). However upon xenobiotic stress, Nrf2 is liberated from Keap-1 permitting its translocation into the nucleus (Copple, 2012). As Nrf2 accumulates in the nucleus, it would seem more appropriate to assess Nrf2 content within this more concentrated organelle rather than the whole cell (Thimmulappa et al., 2007). Human T cells (CD3⁺ T cells: 96 %, data not shown) were treated with increasing concentrations of CDDO-Me as before, nuclear extracts were prepared and nuclear expression of Nrf2 was assessed by western blot. In contrast to the WCL data, results clearly demonstrated that Nrf2 expression is detected both basally and upon CDDO-Me treatment (**Figure 5.2**). Furthermore, a CDDO-Me concentration-dependent increase in Nrf2 expression was observed (**Figure 5.2**). Taken together, these results indicate the increased sensitivity of examining of Nrf2 in the nucleus of human T cells, as even basal Nrf2 levels can be identified through this optimised method. Moreover, the CDDO-Me concentration-dependent increase in Nrf2 expression validated the utilisation of homogenous CD3⁺ human T cells as a suitable *ex vivo* model for examining human variation in iNrf2 expression in response to a known Nrf2 inducer.

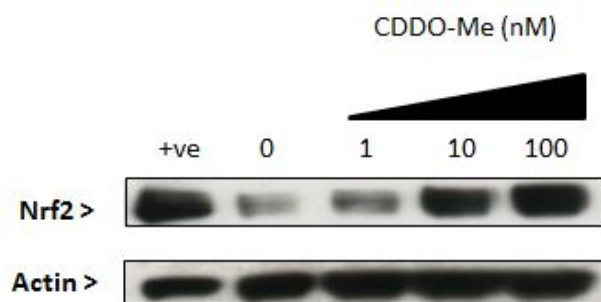


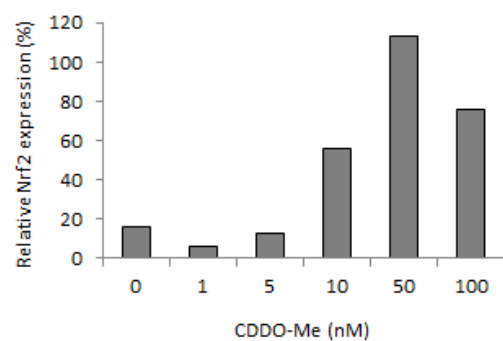
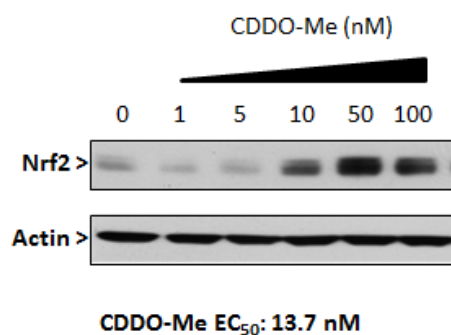
Figure 5.2 CDDO-Me induces nuclear accumulation of Nrf2 in human T cells. Human PHA-stimulated T cells were treated with increasing concentrations of CDDO-Me (0-100nM in 0.1 % DMSO) for 2 h and nuclear fractions were prepared. The nuclear proteins (20 μ g) were subjected to SDS-PAGE, and levels of Nrf2 and the loading control protein β -actin were determined by western blot analysis. HepG2 cells treated with the proteasome inhibitor MG132 (10 μ M) for 2 h, was used as a positive control.

5.2.3 Inter-individual variation in Nrf2 adaptive response to CDDO-Me is present in human T cells.

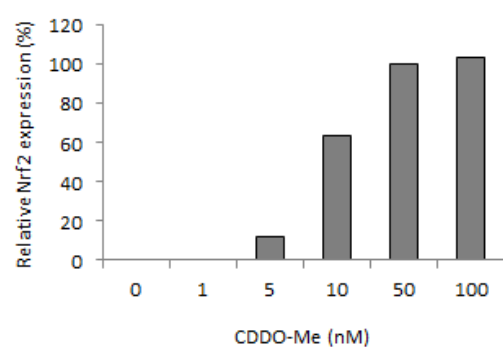
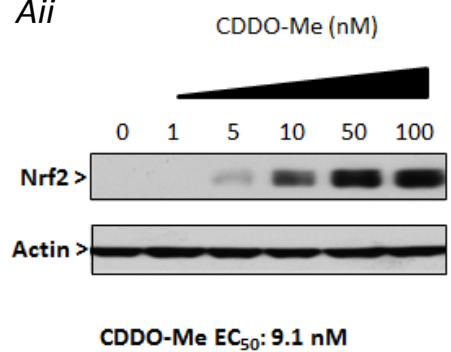
As we have established an optimised protocol for the detection of Nrf2 in human T cells, we next sought to determine whether differences could be observed in basal and CDDO-Me inducible Nrf2 expression between individuals. Donor T cells were subsequently treated with CDDO-Me for 2 h and nuclear fractions were assessed for Nrf2 expression via western blot. Nrf2 protein bands were quantified by densitometry and Nrf2 was expressed as a percentage relative to β -actin (**Figure 5.3A-B**). To calculate the half maximal effective concentration (EC_{50}) of CDDO-Me for each donor, the densitometry values (**Figure 5.3Ai-xi**) were converted into percentage of maximal response (highest percentage of Nrf2 expression exhibited by each donor was set at 100% maximal response). A concentration-response curve (% response versus CDDO-Me concentration) was then plotted for each individual and EC_{50} determined (data not shown). As shown in **Figure 5.2Ai-xi**, clear trends were apparent within the obtained data. Donors could be categorised based on their basal expression of Nrf2 (densitometry), the magnitude of the levels of Nrf2

expressed (densitometry), and how sensitively the donors responded to CDDO-Me (EC_{50}). Results revealed that T cells derived from donors 1 (**Figure 5.3Ai**), 3 (**Figure 5.3Aiii**), 4 (**Figure 5.3Aiv**) and 6 (**Figure 5.3Avi**) exhibited enhanced basal levels of nuclear Nrf2 (D1: 16.4 %, D3: 37.2 %, D4: 19.3 %, D6: 15.5 %). Donor 4-derived T cells displayed the highest magnitude of Nrf2 expression as illustrated in **Figure 5.3Aiv** (100nM CDDO-Me: 192.9 %). In contrast, D5 T cells exhibited the lowest magnitude of Nrf2 expression (**Figure 5.3Av**, 5nM CDDO-Me: 55.9 %). The EC_{50} value was used to determine variation in the sensitivity of the individual's adaptive Nrf2 system to CDDO-Me. The mean EC_{50} value calculated for all 11 subjects was 16.8 ± 9.1 nM ranging from 6nM (**Figure 5.3Aiii**, D3) to 30.1nM (**Figure 5.2Avi**, D6). Overall, D4-derived T cells exhibited the highest sensitivity to CDDO-me induction of nuclear Nrf2 accumulation; whereas D6 T cells displayed the least sensitivity to CDDO-Me Nrf2 induction (**Figure 5.2Aiv and vi**). The order of most sensitive to least sensitive donor-derived T-cells was D3 > D4 > D2 > D7 > D8 > D1 > D9 > D5 > D11 > D10 > D6 (**Figure 5.2A**). Evaluation of the collated data revealed that Nrf2 was significantly induced from baseline at CDDO-Me concentrations of 10nM and above (**Figure 5.3B**, $p < 0.01$). Taken together, our results clearly demonstrate that variation exists between individuals in their Nrf2 adaptive response to CDDO-Me. In light of this, it was necessary to assess a functional output of this Nrf2 inter-individual variation through examination of Nrf2 downstream gene expression.

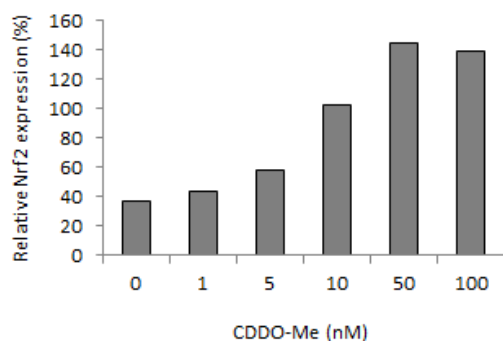
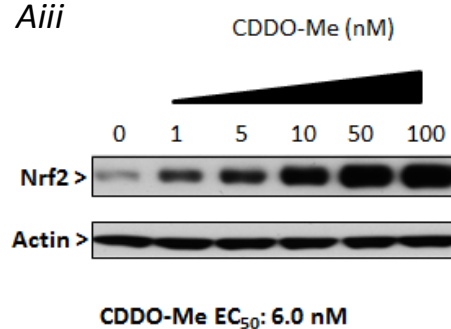
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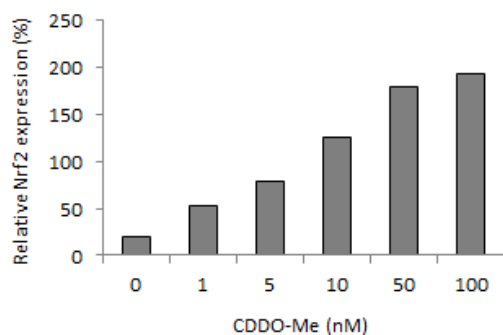
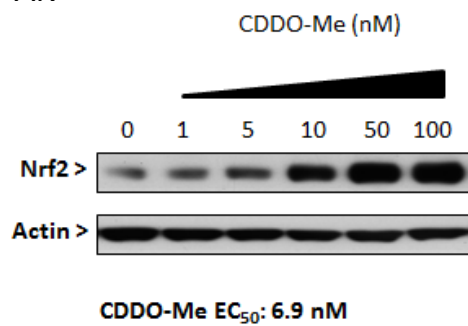
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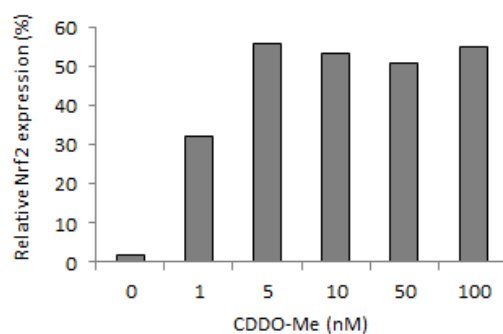
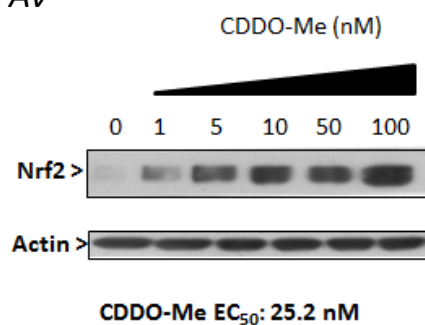
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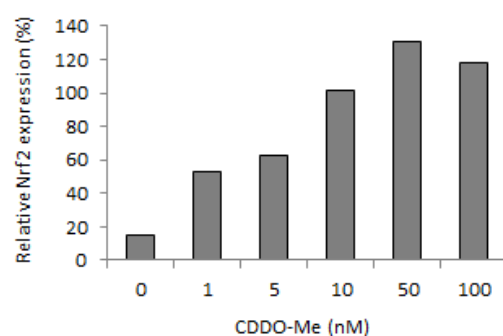
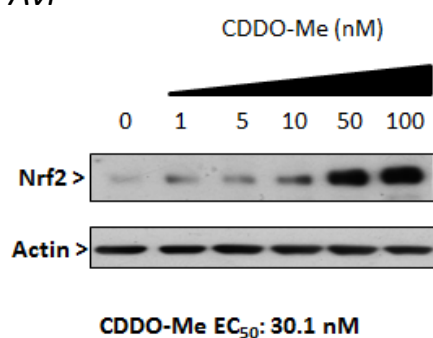
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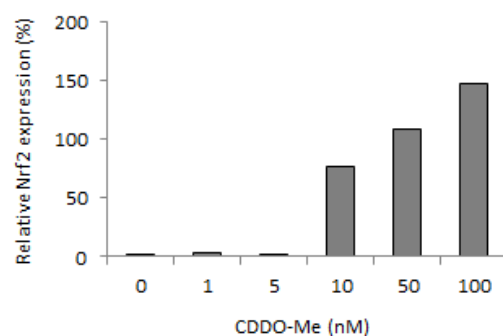
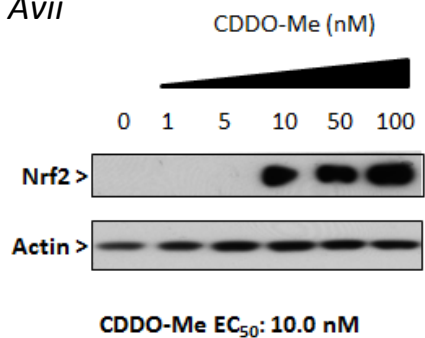
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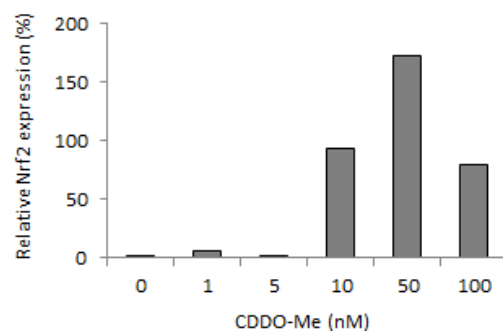
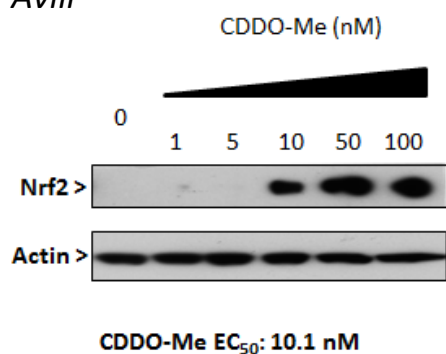
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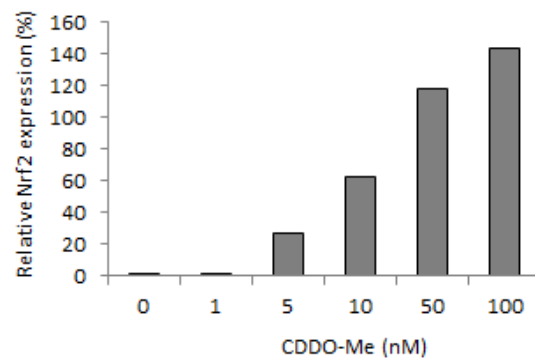
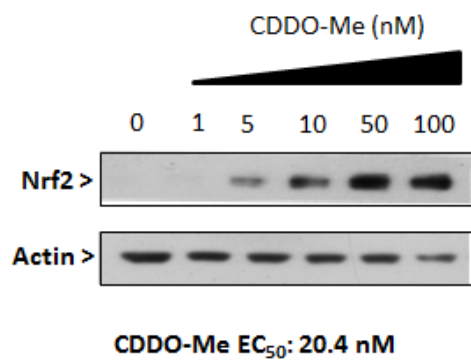
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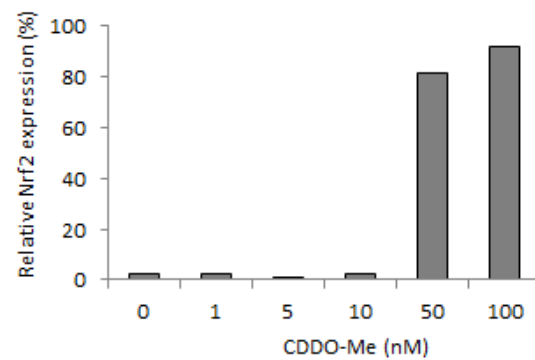
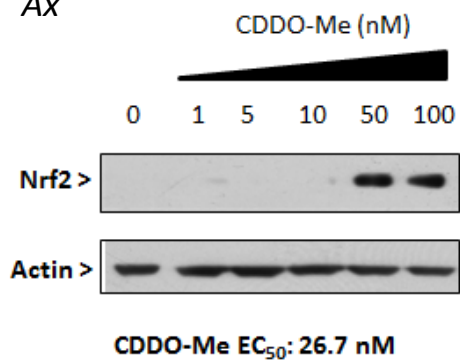
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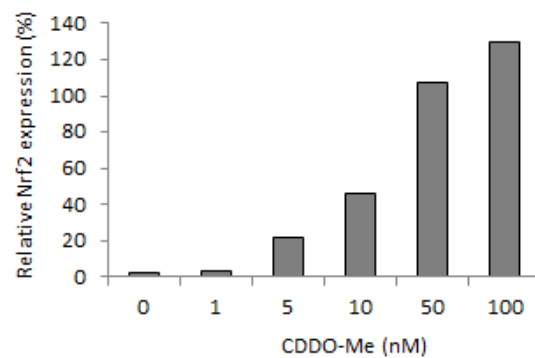
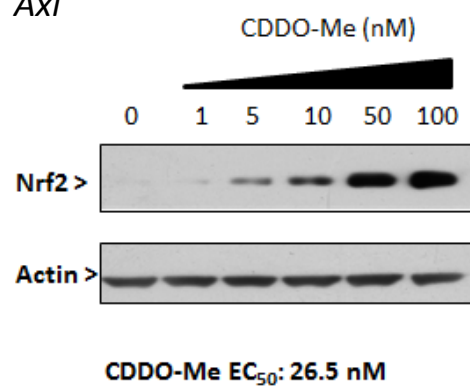
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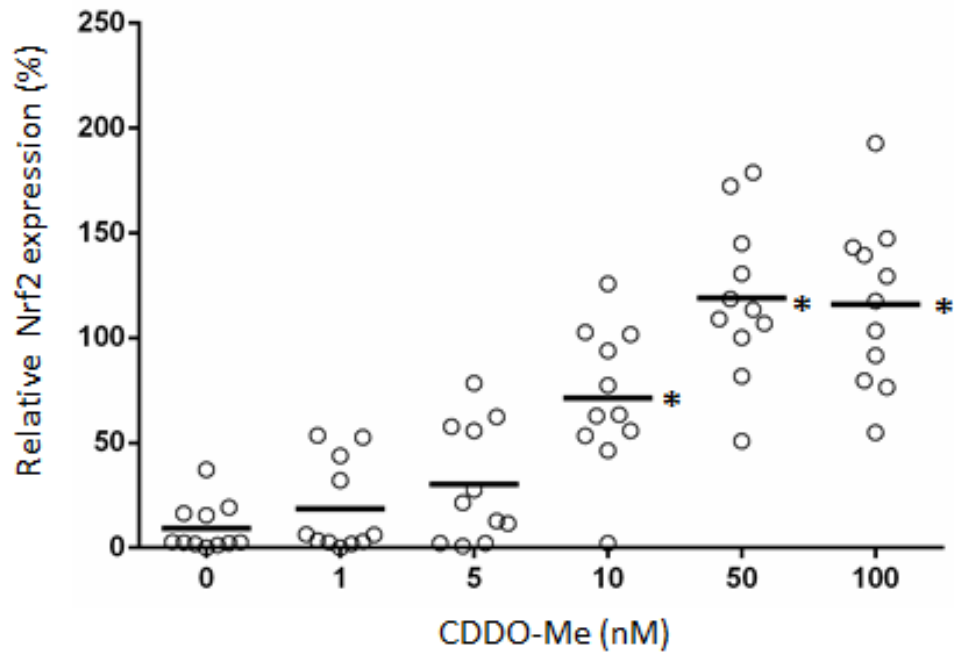


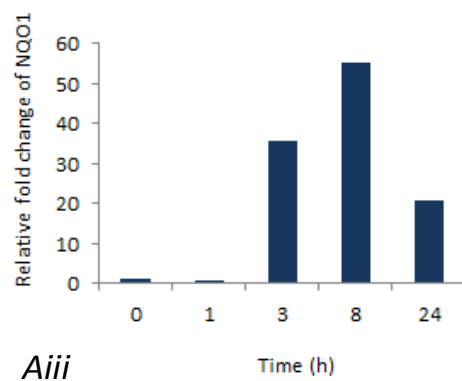
Figure 5.3 Inter-individual variation in Nrf2 adaptive response to CDDO-Me is present in human T cells. Human CD3⁺ PHA-stimulated day 5 T cells derived from donors **Ai** D1, **ii.** D2, **iii.** D3, **iv.** D4, **v.** D5, **vi.** D6, **vii.** D7, **viii.** D8, **ix.** D9, **x.** D10 and **xi.** D11 were treated with increasing concentrations of CDDO-Me (0-100nM in 0.1 % DMSO) for 2 h and nuclear fractions were prepared. The nuclear proteins (20μg) were subjected to SDS-PAGE; levels of Nrf2 and the loading control protein β -actin were determined by western blot analysis. Nrf2 protein bands were quantified by densitometry and Nrf2 was expressed as a percentage relative to β -actin. To calculate the half maximal effective concentration (EC_{50}) of CDDO-Me for each donor, the densitometry values were converted into percentage of maximal response so that the highest percentage of Nrf2 expression exhibited by each donor was set to 100% maximal response. A concentration-response curve (% response versus CDDO-Me concentration) was then plotted for each individual and EC_{50} determined. **B.** Collated densitometry values for donors 1-11 in response to increasing concentrations of CDDO-Me. Treatment groups were compared to the untreated control (0nM CDDO-Me). Statistical significance was assessed using the one way analysis of variance (ANOVA) (*, $p < 0.05$).

5.2.4 Inter-individual variation in Nrf2 downstream NQO1 gene expression is present in human T cells

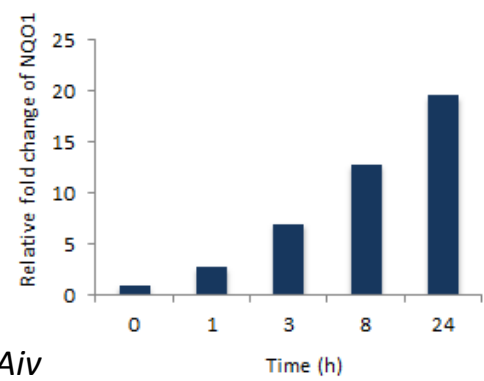
Once Nrf2 translocates into the nucleus, it upregulates the transcription of an array of cytoprotective and antioxidant genes which are involved in the orchestration of the Nrf2-mediated cellular defence (Copple, 2012). The Nrf2-regulated gene NAD(P)H:quinine oxidoreductase 1 (NQO1) encoding the NQO1 enzyme, plays a crucial role in counterbalancing xenobiotic-mediated cellular stress, through the detoxification of quinone compounds and their corresponding reactive metabolites (Copple et al., 2010). Nrf2-dependent upregulation of NQO1 gene expression, has been identified in a variety of human cell types including hepatocytes (Keum et al., 2006), keratinocytes (van den Bogaard et al., 2013), PBMCs and neutrophils (Thimmulappa et al., 2007). Moreover, inter-individual variation in CDDO-Me induction of NQO1 gene expression was exhibited in human PBMCs (Thimmulappa et al., 2007). It is unknown whether CDDO-Me induces Nrf2-dependent upregulation of NQO1 expression in human T cells, and if variation in the induction of NQO1 is prevalent. We therefore sought to assess the effect of CDDO-Me on Nrf2-dependent downstream NQO1 expression, in day 5 PHA-stimulated CD3⁺ T cells human T cells derived from a small group of 6 donors. In order to investigate the latter, an appropriate concentration of CDDO-Me was initially selected that would significantly induce Nrf2 and potential downstream NQO1 expression in human T cells. This was based on the collated data shown in **Figure 5.3B**, which illustrated that significant induction of Nrf2 was attained at concentrations of CDDO-Me from 10nM and above. It would thus seem reasonable to choose 10nM as a candidate concentration for gene expression studies. However, CDDO-Me-mediated Nrf2 induction was not detected in D10-derived T cells at this particular concentration (**Figure 5.2Ax**). For this reason, the higher 50nM CDDO-Me concentration was selected to ensure Nrf2 induction in all subsequent donors. Donor T cells were treated with CDDO-Me (50nM) and IL-2 (for T-cell survival) over a 0-24 h time course. Ribonucleic acid was then isolated and reverse transcribed into cDNA. NAD(P)H:quinine oxidoreductase 1 and housekeeping gene GAPDH mRNA levels were measured within these samples by RT-PCR. The NQO1 mRNA

data was normalised to GAPDH and converted into fold increase in NQO1 expression compared to untreated cells. Results illustrated that differences were observed between donors T cell CDDO-Me-induced NQO1 levels, in terms of the magnitude of NQO1 expression and the time at which maximal NQO1 mRNA levels were achieved (**Figure 5.4Ai-vi**). Within the small donor group, D15 exhibited the highest levels of NQO1 gene expression after 8 hours of CDDO-Me treatment (**Figure 5.4Avi**, 8h: 75-fold increase in NQO1 mRNA expression). Furthermore, high NQO1 induction was observed as early as 1 hour post CDDO-Me treatment in comparison to the other donors (**Figure 5.4Avi**, 1hr: 12-fold NQO1 increase). In contrast, minimal induction of NQO1 was observed for D14 and D12-derived T cells over the 24 hour time course; maximal NQO1 expression achieved at 24 and 8 hours post-CDDO-Me treatment for D12 and D14, respectively (**Figure 5.4Aiii**, 6-fold NQO1 mRNA increase; **Figure 5.4Av**, 4-fold NQO1 mRNA increase). Similarly to D15, D13-derived T cells attained maximal NQO1 levels after 8 hours of CDDO-Me treatment (**Figure 5.4Aiv**, 22-fold mRNA NQO1 increase); whereas highest levels of NQO1 were reached at as early as 3 hours post CDDO-Me treatment for D1 (**Figure 5.4Ai**, 30-fold NQO1 mRNA increase). A clear time-dependent increase in CDDO-Me-induced NQO1 expression was observed for D9-derived T-cells (**Figure 5.4Aii**). Assessment of the collated data, illustrated that significant induction of NQO1 expression by CDDO-Me was observed after 3 hours of dosing and above, when compared to the untreated cells (**Figure 5.4B**, $p < 0.05$). Taken together, our findings clearly demonstrate that variation is also prevalent in Nrf2-regulated downstream NQO1 gene expression in response to CDDO-Me in human T cells.

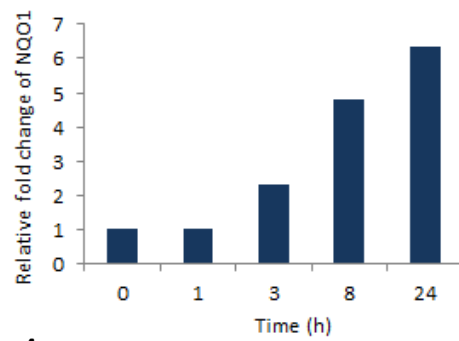
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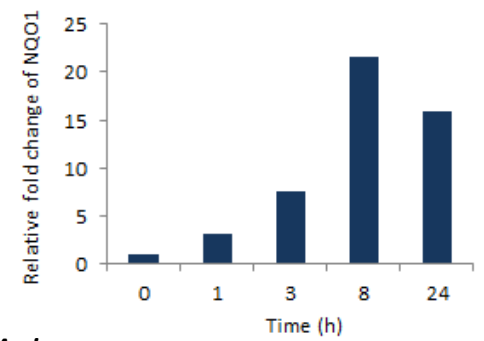
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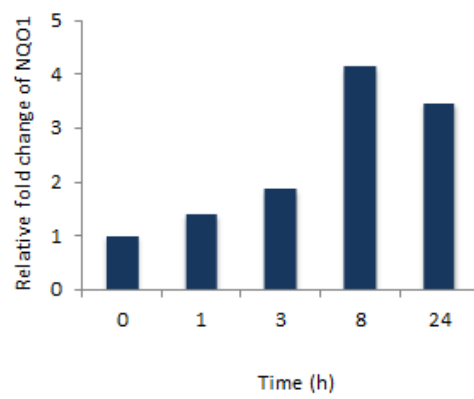
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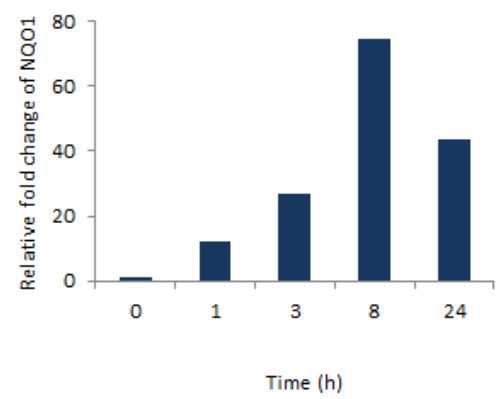
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A scatter plot showing the relative fold change of NQO1 over time (0, 1, 3, 8, 24 hours) after treatment with 10 μ M H₂O₂. The y-axis is labeled "Relative fold change of NQO1" and ranges from 0 to 80. The x-axis is labeled "Time (h)" and has categories 0, 1, 3, 8, and 24. Data points are represented by open circles. Horizontal black bars indicate the mean value for each time point. Asterisks (*) above the bars at 3, 8, and 24 hours indicate statistical significance compared to the 0-hour control.

Time (h)	Relative fold change of NQO1 (approximate values)
0	~1, ~1, ~1, ~1, ~1, ~1, ~1, ~1, ~1, ~1
1	~3, ~1, ~1, ~1, ~1, ~1, ~12
3	~12, ~27, ~29, ~6, ~6, ~1, ~1, ~1
8	~23, ~21, ~21, ~13, ~4, ~4, ~4
24	~18, ~19, ~19, ~43, ~4, ~6

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5.3 DISCUSSION

Extensive inter-individual variability is prevalent in human responses to xenobiotic exposure. This can range from drug adaptation and tolerance to severe life threatening idiosyncratic ADRs. Evaluation and prediction of these idiosyncratic ADRs proves extremely difficult due to this human variability, which is highly influenced by a myriad of host-specific factors such as gender, age, underlying disease status and genetic makeup (Severino and Del Zompo, 2004). During drug metabolism, harmful reactive metabolites and ROS are generated which have the potential to induce cellular necrosis and onset of disease (Park et al., 2005a). The transcription factor Nrf2 plays a pivotal role in the induction of phase II detoxifying and antioxidant enzymes in response to xenobiotic-oxidative insult, thus is indispensable for the maintenance of cellular redox homeostasis and prevention of drug-induced cellular damage (Copple et al., 2010). A plethora of evidence using human cell lines has highlighted the involvement of the Nrf2 pathway in cellular protection against xenobiotic stress. Examples include curcumin-mediated protection against arsenite-induced cytotoxicity in human keratinocyte HaCaT cells (Zhao et al., 2013), and sulforaphane-mediated diminution of microcystin-induced cytotoxicity in HepG2 cells (Gan et al., 2010). This has also been further exemplified in studies using primary murine hepatocytes (Shinkai et al., 2006) and Nrf2 deficient mice, the latter of which are more susceptible to xenobiotic-induced organ toxicity (Enomoto et al., 2001; Cho et al., 2004; Goldring et al., 2004; Aleksunes et al., 2010; Kikuchi et al., 2010; Kay et al., 2011). Xenobiotic-mediated activation of Nrf2 has been detected in a variety of human cell types including hepatocytes, PBMCs, neutrophils, alveolar type II cells and keratinocytes (Keum et al., 2006; Thimmulappa et al., 2007; Messier et al., 2013; van den Bogaard et al., 2013). However, the role of Nrf2 in protection against drug-induced toxicity has not been exclusively explored in humans. Furthermore, it has been suggested that the Nrf2 adaptive cellular defence pathway is also subject to inter-individual variation in response to Nrf2 inducers in humans. (Thimmulappa et al., 2007; Boettler et al., 2011b). However, these findings were not extensive and thus required further reaffirmation. In light of this, several questions remain unanswered; does variation

in Nrf2 adaptive response to xenobiotic stress exists in humans? And is disparity in an individual's susceptibility to drug toxicity associated with variation in the Nrf2 adaptive response? To begin answering these complex questions it must first be established that inter-individual variation exists in the Nrf2 adaptive system in response to a known Nrf2 probe. The present study endeavoured to address the latter, by establishing a method to assess variation in Nrf2 adaptive system to a chemical inducer of Nrf2 in human T cells, using the oleanolic acid derivative, CDDO-Me. This novel synthetic triterpenoid which exhibits anti-inflammatory, anti-proliferative and anti-oxidant properties *in vivo* (Auletta et al., 2010; Li et al., 2010a; Kulkarni et al., 2013), is recognised as a potent inducer of the Nrf2 pathway (Yates et al., 2007). Therefore it posed a likely candidate for manipulating Nrf2 levels within our model. Assessment of human variation was achieved by isolating PBMCs from the blood derived from a small group of healthy subjects. Blood-derived immune cells represent an accessible source of physiologically relevant cells, which can be easily utilised for *ex vivo* human Nrf2 induction studies (Thimmulappa et al., 2007; Boettler et al., 2011b). Therefore, they are an ideal model for the present chapter. The PBMCs were subsequently activated with the human T cell mitogen PHA to enable expansion of a homogenous CD3⁺ T cell population. The use of a homogeneous population is advantageous in such studies as additional cell-based variability (observed with heterogeneous populations) is avoided. Donor-derived CD3⁺ T cell blasts were subsequently treated with increasing concentrations of CDDO-Me for 2 hours to allow for induction of Nrf2 expression. It has been previously shown that Nrf2 can be detected within total cell proteins in human hepatocytes, both basally and upon butylated hydroxyanisole (BHA) treatment (Keum et al., 2006). Therefore, we initially investigated whether CDDO-Me-induced Nrf2 protein expression could be identified within the WCL of human T cells, through western blot analysis. Results illustrated that Nrf2 could not be detected in whole cells. As Nrf2 translocates into the nucleus under conditions of oxidative stress, we reasoned that Nrf2 would be easily detected in this smaller and more concentrated location. Accordingly, we determined the basal and CDDO-Me inducible levels of Nrf2 within the T cell nucleus. Our results illustrated that Nrf2 protein expression can be identified both basally and upon concentration-

dependent CDDO-Me induction within the nuclear fraction of human T cells. In support of our finding, nuclear Nrf2 expression was detected in the nuclear fraction of human PBMCs using a CDDO-Me analogue, CDDO-Im (Thimmulappa et al., 2007). This optimised human *ex vivo* experimental model; therefore, provided an appropriate platform by which to subsequently examine variation in Nrf2 adaptive response to CDDO-Me in a small group of healthy individuals. Assessment of inter-individual variability in donor-derived T cells was based upon basal Nrf2 expression, the magnitude of the Nrf2 response and the overall sensitivity of the Nrf2 system to CDDO-Me-simulated xenobiotic stress. It was apparent that four out of eleven donors exhibited enhanced basal Nrf2 levels. Furthermore, the extent of Nrf2 induction mediated by CDDO-Me differed greatly for each donor. With reference to the sensitivity of the Nrf2 system, as evaluated by the donor's distinctive CDDO-Me EC₅₀ value, a trend emerged in the spread of EC₅₀ values. The EC₅₀ value range could be subsequently divided into quartiles, so that the lowest (6-12nM) and highest (24-30nM) quartile corresponded to donors with low and high EC₅₀ values, respectively. Therefore, donors could be classified into high (EC₅₀ value < 12 nM), intermediate (EC₅₀ value 12-24 nM) and low (EC₅₀ value > 24 nM) responding groups. Donor 2, D3, D4, D7 and D8 fell into the high responding group; D1 and D9 in the intermediate category; whilst D5, D6, D10 and D11 were found to be low responders corresponding to the highest EC₅₀ quartile. Although the numbers of donors were limited, it appeared that individuals generally fell within the high and low responding groups. What factors could account for this observed disparity in human Nrf2 adaptive response to CDDO-Me in this particular model? Marked inter-individual variability is prevalent in human PBMC responses to PHA stimulation (Verheugen et al., 1997), which could ultimately impact on the Nrf2 response exhibited in the human T cell blasts. Within humans, it has been established that dietary intake of 5-O-caffeoylquinic acid-rich coffee, sulforaphane-containing broccoli preparations, resveratrol (the active polyphenol compound present in grapes and red wine) and curcumin (active ingredient of turmeric spice) have shown to activate Nrf2 signalling in PBMCs (Wu et al., 2010; Boettler et al., 2011b; Harvey et al., 2011). Furthermore, cigarette smoke, coal, tar, and diesel exhaust particles have also been shown to alter Nrf2 expression in human alveolar cells,

keratinocytes and PBMCs, respectively (Messier et al., 2013; van den Bogaard et al., 2013; Yamamoto et al., 2013). It would therefore seem plausible that additional dietary and environmental xenobiotic exposure, would impact on the individual's Nrf2 basal levels and adaptive response to CDDO-Me. Under conditions of oxidative stress, Nrf2 accumulates in the nucleus where it binds to the ARE in the promoter region of Nrf2-regulated cytoprotective and antioxidant genes including GST, HO-1 and NQO1, resulting in their transactivation and induction of cellular defence (Copple, 2012). NQO1 is responsible for the nullification of quinone compounds and their derivatives (Copple et al., 2010). Previous studies using Nrf2 deficient mice have highlighted the critical role of Nrf2 in the transcription of NQO1 gene expression in response to xenobiotic stress (Kwak et al., 2001; Rangasamy et al., 2004; Xu et al., 2008). Furthermore, it has been established that activation of Nrf2 results in the upregulation of NQO1 gene expression in murine primary hepatocytes and activated CD3/CD28 T cells (Keum et al., 2006; Rockwell et al., 2012). Within humans, BHA-induced and coal tar-induced activation of Nrf2 results in upregulation of NQO1 in hepatocytes and keratinocytes, respectively (Keum et al., 2006; van den Bogaard et al., 2013). As inter-individual variation was observed in Nrf2 protein expression in human T cells, we hypothesised that disparity would also be exhibited in downstream gene expression of NQO1 between donors in response to CDDO-Me. Generally our results revealed a time-dependent increase in CDDO-Me-mediated induction of NQO1. This has also been illustrated in BHA-treated human hepatocytes (Keum et al., 2006). It was observed that individuals varied in their overall magnitude of NQO1 gene expression and time at which peak NQO1 expression was achieved, in response to CDDO-Me. From the present study we cannot correlate the differences in individual's Nrf2 expression to downstream changes in NQO1 expression, as different donors were used per experiment. However, we can still ascertain that inter-individual variation is present in CDDO-Me-mediated NQO1 gene expression in human T cells. A previous study has revealed that variation in Nrf2 and NQO1 expression was prevalent in the lungs of healthy individuals. However, increased Nrf2 expression did not necessarily amount to increased NQO1 expression (Malhotra et al., 2008). It would therefore be valuable to simultaneously investigate levels of Nrf2 and NQO1 gene expression in

each individual to facilitate correlation between CDDO-Me-mediated Nrf2 induction and its subsequent downstream events. An extensive examination of Nrf2-downstream gene expression profile including HO-1 and GCLC (Keum et al., 2006) in response to CDDO-Me per individual, would further support the concept that variation is exhibited in Nrf2 adaptive system in response to an Nrf2 inducer. It has also recently been suggested that the nrf2-regulated gene expression of aldo-keto reductase (AKR) could be used as a marker of xenobiotic-induced Nrf2 activity in human monocytes (Jung et al., 2013). Therefore it would be interesting to assess levels of AKR in our human T cell model. It would also be worthwhile to evaluate the effect of a panel of Nrf2 activators such as curcumin, sulforaphane, BHA and tBHQ (Keum et al., 2006; Wu et al., 2010; Rockwell et al., 2012) on this *ex vivo* model, to assess whether the variation in Nrf2 adaptive response is still prevalent, and if it is specific to each type of Nrf2 probe.

It is recognised that genetic variation in phase I drug metabolising CYP450 enzymes are associated with increased susceptibility to idiosyncratic ADRs. For example, individuals carrying polymorphisms in genes encoding CYP2E1 are more susceptible to isoniazid-induced hepatitis. This is a result of enhanced phase I oxidation of isoniazid metabolic intermediates into hepatotoxic compounds (Huang et al., 2003). Similarly, genetic variations in the human leukocyte antigen (HLA) (also known as MHC molecule), which is crucial for the APC presentation of drug-derived antigens to T cells, are strongly associated with immune-mediated ADRs e.g. (HLA)-B*57:01 association with flucloxacillin-induced liver injury (Monshi et al., 2013). Indeed, genetic polymorphisms in humans have also been identified in the promoter region of the Nrf2 gene. These have been associated with reduced Nrf2 transcriptional activity, correlating with an increased risk of acute lung injury in humans (Marzec et al., 2007). Other Nrf2 polymorphisms have correlated with *Helicobacter pylori*-induced gastritis (Arisawa et al., 2007) and ulcerative colitis (Arisawa et al., 2008). It would be interesting to examine the genotype of each donor to observe whether differences in their individual Nrf2 adaptive system correlate with polymorphisms in the Nrf2 gene itself.

The works presented in this chapter has established a means by which to examine human variation in Nrf2 adaptive system in an accessible source of physiologically relevant cells. We next need to investigate whether this inter-individual variability correlates with individual susceptibility to drug toxicity. Thus, could drug-induced changes in Nrf2 in blood-derived immune cells be used as a potential biomarker to predict drug toxicity in inaccessible organs such as the liver in susceptible individuals? In order to address this important issue it is imperative to correlate drug-induced Nrf2 expression in primary human hepatocytes with Nrf2 levels in the blood derived from the same donor. Our currently established *ex vivo* model is not without its limitations; however, for this more detailed type of correlative assessment. The donor-derived T cells have been initially stimulated with PHA and grown out for 5 days; therefore, could not be compared to freshly isolated hepatocytes from the same donor. In this case, it would be more accurate to investigate Nrf2 levels in freshly isolated PBMCs (Thimmulappa et al., 2007). It therefore would be valuable to treat donor hepatocytes and their corresponding PBMCs with various concentrations of a known hepatotoxic drug such as acetaminophen (Goldring et al., 2004), and assess correlation in levels of Nrf2 expression in both cells. A previous study has highlighted the importance of using PBMCs to measure the activity of particular drug metabolising enzymes, for the prediction of liver toxicity in patients undergoing chemotherapy (Lu et al., 1993). Deficiency in PBMC dihydropyrimidine dehydrogenase enzyme activity, which is responsible for the catabolism of 5-fluorouracil, correlated with 5-fluorouracil-induced liver toxicity in cancer patients (Lu et al., 1993). In spite of this, the utilisation of human PBMCs as a suitable model to correlate drug-induced Nrf2 levels to hepatocytes would also be problematic, due to the heterogeneous nature of PBMCs in comparison to the homogenous hepatocytes. Moreover, the composition of macrophages, monocytes and lymphocytes within PBMCs differs greatly for each individual. Human hepatocytes are generally derived from patients with liver cancer and thus would be subject to chemotherapeutic drugs. Chemotherapeutics are known to cause leukopenia (Lu et al., 1993); therefore, the numbers of PBMCs attained from each patient may be extremely limiting. An alternative source of amenable immune cells to the PBMCs would be the

neutrophils. These immune cells are extremely abundant in the blood, easily isolated and are homogenous in nature. Moreover, nuclear Nrf2 levels can be detected in human neutrophils (Thimmulappa et al., 2007) thus pose an attractive candidate for the aforementioned predictive liver drug-toxicity model.

CHAPTER SIX

FINAL DISCUSSION

6.1 DISCUSSION

The body has evolved a cellular adaptive defence system which counterbalances a myriad of oxidative and electrophilic stressors such as environmental diesel exhaust fumes and xenobiotics. The functionality of this cellular defence system is principally governed by the activity of the redox-sensitive transcription factor Nrf2 (Copple, 2012). In response to oxidative stress, Nrf2 induces the transcription of a battery of cytoprotective and antioxidant genes e.g. GCLC, GST and NQO1, resulting in the nullification of oxidative or chemical insult and maintenance of cellular redox homeostasis (Copple, 2012). It is recognised that Nrf2 plays a protective role in a variety of murine disease models including sepsis, acute lung injury, asthma and EAE, all of which are immune-mediated (Rangasamy et al., 2005; Thimmulappa et al., 2006a; Reddy et al., 2009a; Johnson et al., 2010). To this end, it emerged that Nrf2 plays a prominent role in the regulation of the immune responses. However, its precise role in DCs and T cells is poorly understood (Ma et al., 2006; Williams et al., 2008; Rangasamy et al., 2010; Rockwell et al., 2012).

In light of this, the central aim of the present thesis was to further elucidate the role of Nrf2 in murine DC and T cell immune function, through the utilisation of the Nrf2 deficient mouse experimental model.

With reference to DCs, this study demonstrated that Nrf2 deficiency in iDCs resulted in lowered GSH levels, enhanced iDC co-stimulatory receptor expression, impaired endocytic and phagocytic capacity, and increased iDC-mediated antigen-specific CD8 T cell stimulatory capacity. Furthermore, artificially lowering GSH levels in the iDCs did not recapitulate the Nrf2 deficient iDC phenotype. (Aw Yeang et al., 2012). Immature DCs constitutively present self-peptides to CD8 T cells, and instruct the deletion of peripheral autoreactive T-cells that have escaped from selection processes in the thymus. Hence, they play a crucial role in the maintenance of peripheral CD8 T cell tolerance (Kurts et al., 1997; Kurts et al., 1998; Steinman et al., 2000). It has been previously shown that increases in co-stimulatory receptor expression in iDCs causes a break in peripheral T cell tolerance, through the prolongation of T cell activation and enhancement of

adaptive immune responses (Hawiger et al., 2001). Therefore, it would be reasonable to propose that in the absence of infection, the Nrf2 deficient iDCs could present self-antigens to CD8 T cells in such a context that (instead of instructing autoreactive T cell deletion or anergy) may result in the induction of inappropriate CD8 T cell activation, adaptive immune responses and autoimmunity. The observations that aged Nrf2 deficient mice manifest enhanced lymphoproliferation and autoimmune-like symptoms, further emphasises the underlying role of Nrf2 in the regulation of T cell tolerance and autoimmunity (Ma et al., 2006). Nrf2^{-/-} iDCs also exhibited a greater capacity to extract and cross-present cell-associated antigens derived from dying cells to CD8 T cells. However, the increased co-stimulatory receptor expression exhibited by the Nrf2 deficient iDCs cannot be ruled out as the driving force behind the increased cross-presentation observed. We have recently established that Nrf2^{-/-} DCs produced high levels of ROS in relation to their wild type counterpart (Aw Yeang et al., 2012). Reactive oxygen species production through TLR9 signalling in DCs, promotes processing and cross-presentation of intracellular *Salmonella* bacterial antigens to CD8 T cells resulting in increased IFN γ production (Lahiri et al., 2010). Although we have concluded that enhanced co-stimulatory receptor expression exhibited in the Nrf2 deficient is independent of ROS levels (Al-Huseini et al., 2013), this does not rule out the possibility that the DC-mediated ROS production may be a contributing factor towards the increased cross-presentation. It would therefore be valuable to treat the DCs with vitamin C and E (Al-Huseini et al., 2013) and subsequently assess their cross-presentation capacity of necrotic cell-associated antigens to CD8 T cells. In general, necrotic cells which release mediators such as High-Mobility Group Box 1 Protein (HMGB1) can activate TLR4 and TLR2. Toll-like receptor engagement initiates DC maturation ultimately resulting in DC-driven CD8 T cell immunity. In contrast, apoptotic cells which are immunologically silent and thus do not induce DC maturation, facilitate iDC-mediated CD8 T cell tolerance (Kurts et al., 1998; Ip and Lau, 2004; Miyake and Yamasaki, 2012; Zelenay et al., 2012). It would therefore be valuable to examine the consequence of loss of Nrf2 in DC-mediated cross-presentation of antigens derived from apoptotic cells to CD8 T cells, to see whether enhanced CD8 T cell stimulatory capacity would also be observed within this self-

antigenic context. Finally in this study, we highlighted that loss of Nrf2 in LPS-activated DCs resulted in lowered TNF α and IL-12 production. However, we found no differences in IL-2 secretion between Nrf2^{+/+} and Nrf2^{-/-} DCs upon LPS stimulation. This suggests that Nrf2 deficiency induces a lowered Th1 cytokine profile within a DC-mediated Th1 polarising context (Agrawal et al., 2003). Therefore during a bacterial infection, it could be said that Nrf2 deficient DCs may secrete less TNF α and IL-12, resulting in a diminished capacity to prime neighbouring DCs to induce Th1 responses, leading to a reduction in clearance of infection. From a tolerance perspective, it has been previously shown that TNF α -producing iDCs induce the differentiation of antigen-specific CD4⁺ IL-10-producing regulatory T cells which are known to dampen inappropriate immune responses (Hirata et al., 2010). Therefore, as the Nrf2 deficient iDCs secrete less TNF α , it may be the case that these DCs fail to induce the differentiation of such regulatory T cells, resulting in a diminution of T cell tolerance. Previous studies have demonstrated that under an allergic environment known to induce Th2 responses, Nrf2 DCs exhibited an altered Th1/Th2 cytokine profile skewed towards the Th2 phenotype (Williams et al., 2008; Rangasamy et al., 2010). This highlights a pivotal role for Nrf2 in the regulation of DC Th1/Th2 cytokine production. To further assess the role of Nrf2 in Th1/Th2 differentiation, it would be worthwhile to examine the consequences of this altered DC cytokine secretion on antigen-specific CD4 effector T cell function through the utilisation of OT-II transgenic mouse (Williams et al., 2008). Within this model, the CD4 T cells exclusively express TCR specific for ovalbumin peptide (OVA₃₂₃₋₃₃₉) (Williams et al., 2008; Hirata et al., 2010). The Nrf2^{+/+} and Nrf2^{-/-} DCs could be pulsed *ex vivo* with OVA₃₂₃₋₃₃₉ and injected into the OT-II transgenic mouse. Purified splenic CD4⁺ OT-II T cells could then be re-stimulated with OVA-pulsed wild type DCs and effector Th1/Th2 cytokine production assessed. Furthermore, expression of the master transcription factors T-bet and GATA-3 for Th1 and Th2 cells, respectively (Rockwell et al., 2012), could also be assessed by flow cytometry. The presented study in chapter 3 is not without its limitations. The DCs utilised are BM-derived and thus require 7 days to differentiate into DCs in the presence of GM-CSF. Therefore, these cells are far removed from their normal development in an *in vivo* setting. However, this

method is advantageous in that it allows the assessment of Nrf2 deficiency in uninfluenced DCs, in that they have not received any additional signals from other surrounding cells in their microenvironment.

As DCs are the critical drivers of T cell-mediated immunity, DC-based immunotherapy pioneered by the late Dr. Ralph Steinman, has emerged at the forefront of vaccinology for the potential treatment of chronic infectious diseases and cancer; typified by attenuated T cell responses (Banchereau et al., 2005; Steinman and Banchereau, 2007; Giannopoulos et al., 2010; Li et al., 2012b; Martinet et al., 2012). Generally, DC immunotherapy is based on the utilisation of patient PBMCs and separation of CD34⁺ progenitor cells or monocytes (Banchereau et al., 2005; Li et al., 2012b). These autologous cells are differentiated into DCs and subsequently loaded with viral-specific peptides (in the case of HCV infection) or tumour lysates (in the case for melanoma). The antigen-bearing DCs are then reintroduced into the host in an attempt to boost CD4⁺ and CTL responses (Banchereau et al., 2005; Li et al., 2012b). These investigations have led to the development of the first FDA-approved DC-based cancer vaccination for the treatment of prostate cancer (Hovden and Appel, 2010). Targeting Nrf2 in DCs could prove beneficial for the treatment of a disease settings associated with Th2 dominance such as asthma. Indeed, loss of Nrf2 results in the exasperation of disease severity in a murine model of asthma (Rangasamy et al., 2005). Allergic mechanisms are initially triggered by low concentration allergen exposure, resulting in weak TCR signalling and subsequent DC-mediated Th2 differentiation (Holgate 2012). Effector Th2 cells trigger B cell allergen-specific IgE production, which is secreted throughout the body and binds to high-affinity IgE receptors on the cell surface of basophils and mast cells (Voehringer 2013). Cross-linkage of the FcεR1:IgE complex with multivalent allergens results in mast cell and basophil activation, and degranulation of inflammatory mediators e.g. histamine. These mediators induce an array of maladaptive effector functions such as local inflammation, activation of innate immune cells, mucus overproduction and smooth muscle contractility, ultimately resulting in the onset and maintenance of allergic disease (Pulendran and Artis 2012). A currently adopted method used to

treat allergic disorders including asthma and rhinitis is sublingual immunotherapy (SLIT) (Akdis et al., 2006). This involves the administration of the specific allergen responsible for the patient's symptoms to the oral mucosa, in an attempt to desensitise the subject to the offending allergen (Akdis et al., 2006). Dendritic cells play a pivotal role in the mechanisms underlying SLIT. It has been proposed that the administered allergen is acquired by oral mucosal DCs, which exhibit a pro-tolerogenic phenotype upon maturation (Novak et al., 2011). This is typified by increased IL-10 production and reduced expression of maturation marker CD83 (Novak et al., 2011). Therefore, these tolerogenic DCs can induce the differentiation of allergen-specific Tregs which dampen the dysregulated Th2 response and downstream allergic mechanisms (Novak et al., 2011). Furthermore, targeted silencing of co-stimulatory molecule CD40, using siRNA in OVA allergen-bearing DCs, resulted in the dampening of OVA-induced allergy in sensitised mice (Suzuki et al., 2010). This was a result of increased OVA-specific Tregs and complete abolishment of IL-4 producing effector T cells (Suzuki et al., 2010). Immunosenescence is also associated with DC-mediated skewed Th2 phenotype (Kim et al., 2008). Treatment of aged DCs with sulforaphane has proven to reset the DC intracellular redox status and restore Th1/Th2 balance (Kim et al., 2008). Therefore it may be possible to prime allergic patient DCs *ex vivo* with both the specific allergen and an Nrf2 activator e.g. sulforaphane, in an attempt to further dampen Th2 responses.

Targeting Nrf2 in DCs to enhance phagocytic function could also be exploited in disease settings associated with reduced phagocyte-mediated apoptotic cell clearance. These include SLE and advanced atherosclerosis in cardiovascular disease (Gaipal et al., 2007; Thorp, 2010). However, failed apoptotic cell clearance exhibited in the aforementioned diseases is predominantly due to macrophage phagocytic dysfunction (Ren et al., 2003; Thorp, 2010). Nrf2 activation induced via conditional knockout of Keap-1 in myeloid cells, improved phagocytic and bactericidal capacity of peritoneal macrophages, challenged with *Pseudomonas aeruginosa* in both healthy and septic mice (Kong et al., 2011). Moreover, treatment of alveolar macrophages derived from COPD patients with sulforaphane, resulted in enhanced

bacterial phagocytosis in response to either nontypeable *Haemophilus influenza* or *Pseudomonas aeruginosa* infection, which was associated with Nrf2-regulated downstream upregulation of the scavenger receptor MARCO (Harvey et al., 2011). This further highlights the critical role of Nrf2 in the regulation of innate immune cell phagocytic function.

Previous studies have shown that Nrf2 activity limits proinflammatory cytokine production e.g. TNF α and IL-1 β in a variety of murine models including sepsis, COPD, brain and spinal cord injury, EAE and pulmonary fibrosis (Thimmulappa et al., 2006a; Jin et al., 2008; Johnson et al., 2010; Kikuchi et al., 2010; Kong et al., 2011; Mao et al., 2011; Yageta et al., 2011). One of the main mechanisms by which Nrf2 exerts its anti-inflammatory activity is through counter-regulation of the NF- κ B pathway (Thimmulappa et al., 2006a; Jin et al., 2008; Kong et al., 2011; Mao et al., 2011; Yageta et al., 2011). NF- κ B plays a pivotal role in cell proliferation, survival, and apoptosis, but importantly it is essential for the regulation of the inflammatory response, and orchestration of both the innate and adaptive immune responses (Hayden and Ghosh, 2011). Various stimuli have shown to activate NF- κ B including oxidative, cellular and mechanical stress, bacterial and viral products, inflammatory cytokines and environmental factors e.g. cigarette smoke and various xenobiotics (Pahl, 1999). Under resting conditions, the NF- κ B heterodimer (generally composed of p50 and p65 (RelA) subunits) is inactive within the cytoplasm, forming a complex with members of the inhibitory I κ B proteins such as I κ B α (Hayden and Ghosh, 2011). Upon activation, upstream I κ B kinase (IKK) complexes are activated, which in turn phosphorylate I κ B α , resulting in its ubiquitination and subsequent degradation (Hayden and Ghosh, 2011). This enables NF- κ B to translocate to the nucleus, where it can bind to specific DNA sequences in κ B sites present within NF- κ B related target genes, resulting in their upregulation and initiation of immune responses (Hayden and Ghosh, 2011). These include cytokines e.g. TNF α , IL-1 β , IFN γ , IL-6, IL-2, IL-12, and immunoreceptors such as MHC I, CD80, CD40 (Pahl, 1999; Ahn et al., 2005). It has been proposed that the NF- κ B subunit p65 can directly repress nrf2 transcriptional activity. It inhibits Nrf2 by competitively antagonising CBP binding to Nrf2 thus preventing Nrf2 transactivation, and also through the recruitment of the

co-repressor histone deacetylase 3 (HDAC3) (Liu et al., 2008). Taken together, this highlights the critical role of Nrf2 in the modulation of inflammation.

From a T cell perspective, this study revealed that loss of Nrf2 did not affect the development of CD4⁺CD25⁺ nTreg, mature CD4 and CD8 T cell populations within the thymus. Furthermore, Nrf2 deficiency did not alter the composition of CD4 and CD8 T cell populations within secondary lymphoid organs. It was observed that splenic Nrf2^{-/-} naïve T cells exhibited enhanced ROS generation; associated with low level increases in T cell activation with respect to lowered CD62L and augmented CD44 expression. However, the marginal augmentation of Nrf2^{-/-} naïve T cell activation status did not mount up to increased T cell proliferation in response to antibody stimulation. In contrast, Nrf2 deficient effector T cells exhibited enhanced TCR/CD3 triggered proliferation, associated with increases in the proportion CD4⁺ IFN γ -producing effector T cells upon re-stimulation. The findings from this study concluded that Nrf2 regulated effector CD4 differentiation. In contrast to Nrf2 deficient DCs which induce strong Th2 responses (Williams et al., 2008), loss of Nrf2 in effector T cells resulted in decreased Th2 (IL-5) and increased Th1 (IFN γ) cytokine secretion which has previously been correlated with reduced GATA-3 and enhanced T-bet DNA binding, respectively (Rockwell et al., 2012). CTLA-4 inhibitory co-receptor engagement has been shown to be important for promoting Th1 cytokine production whilst negatively regulating Th2 effector function (Tao et al., 1997; Oosterwegel et al., 1999). Moreover, STAT1 signalling has been shown to be essential for the induction of T-bet expression in murine splenocytes in response to *Toxoplasma gondii* (*T.gondii*) infection, a potent Th1 stimulating antigen (Lighvani et al., 2001). On the other hand, STAT5 (a key downstream transducer in IL-2 signalling) is indispensable for Th2 differentiation (Zhu et al., 2003). Furthermore, STAT6 is important in regulating Th2-facilitated B-cell immunoglobulin class switching from IgG to IgE, a function of which is vital for humoral immune response to parasitic invasion and allergens (Shimoda et al., 1996). In light of this, it would be worth examining the levels of CTLA-4, STAT1, STAT5 and STAT6 expression in the Nrf2^{+/+} and Nrf2^{-/-} splenic effector CD4 T cells to reaffirm Nrf2 regulation of Th1/Th2 differentiation.

Importantly this study has demonstrated that re-stimulated Nrf2^{-/-} effector T cells secrete increased levels of IL-17A and IL-22, a signature cytokine profile indicative of the more recently identified CD4 Th17 cell lineage (Zhu and Qian, 2012). This was also observed under Th17 polarising conditions, further suggesting that loss of Nrf2 predisposes effector Th17 development. The implications of these findings are huge given the pivotal role that Th17 cells play in the pathogenesis of a variety of autoimmune diseases including SLE, RA, MS and in cancer (Zhu and Qian, 2012; Li et al., 2013). Therefore, activating Nrf2 during CD4 T cell differentiation may reduce Th17 induction and disease progression in the aforementioned disease settings. In support of this, CDDO-TFEA-mediated activation of Nrf2 resulted in reduction in IL-17 production and disease severity in murine EAE model (Pareek et al., 2011). The Th17 subset itself is heterogeneous in nature, which depending upon their initial antigen stimulation and contextual microenvironment; they can exhibit differential cytokine signatures. This ultimately influences their pathogenic inflammatory driving potential in autoimmune murine models such as EAE (McGeachy et al., 2007; Zielinski et al., 2012). For example, CD4 T cells cultured from immunised mice in the presence of TGFβ and IL-6 combination, or IL-23 alone, both induce Th17 development *in vitro*. However, TGFβ and IL-6 treatment induces non-pathogenic IL-17⁺IL-10⁺ immunoregulatory T cells, whilst IL-23 treatment gives rise to pathogenic EAE-driving IL-17⁺IFNγ⁺ producing T cells *in vivo* (McGeachy et al., 2007). Similarly, *Candida albicans* and *staphylococcus aureus* antigen-specific human T cells give rise to Th17 cells that co-produce IFNγ and IL-10, respectively (Zielinski et al., 2012). In our findings, loss of Nrf2 did not influence the proportion of IL-17⁺IFNγ⁺ producing T cells. Examination of the proportion of IL-17⁺IL-10⁺ T cells in the Nrf2 deficient mouse would allow us to see whether the Th17 cells present are immunomodulatory rather than pathogenic.

Dendritic cells play a crucial role in instructing Th17 differentiation through the secretion of TGFβ, IL-6 and IL-23 (Mangan et al., 2006; Veldhoen et al., 2006). Dendritic cell secretion of the aforementioned cytokines is highly dependent upon engagement of their cell surface CLRs including dectin 1 and DC-SIGN (Geijtenbeek and Gringhuis, 2009; Huang et al., 2012). For example, zymosan-mediated

activation of dectin-1 in DCs, results in vital PLC γ 2 signalling upstream of NF- κ B and MAPK, resulting in DC IL-6 and IL-23 production (Xu et al., 2009). Deficiency of p38 MAPK in DCs resulted in a decreased and increased production of IL-6 and IL-27, respectively. Interleukin-27 was shown to negatively regulate of Th17 differentiation, resulting in a significant amelioration of EAE disease severity (Huang et al., 2012). Furthermore, knocking out p38 MAPK resulted in a reduction in CD86 expression in DCs, which is necessary for efficient co-stimulatory signal transduction, further impeding Th17 development (Huang et al., 2012). We have recently demonstrated that enhanced maturation phenotype in Nrf2 deficient DCs is associated with enhanced p38 MAPK phosphorylation (Al-Huseini et al., 2013). In this respect, it would be useful to examine the influence of Nrf2 deficiency in DCs on Th17 differentiation. The presented work on the impact of loss of Nrf2 in murine DC and T cell immune function is summarised in **Figure 6.1**.

Although utilisation of the Nrf2 deficient mouse model is extremely informative for examining DC and T cell function, it is not without its limitations. The global ablation of the *nrf2* gene influences several other signalling pathways and transcription factors including MAPK and NF- κ B (Al-Huseini et al., 2013) Therefore, we cannot say for certain that the phenotype exhibited by the deficient mouse in the particular cell type examined is solely due to the Nrf2 gene itself, but rather a consequence of e.g. increased NF- κ B signalling as observed in the inflammatory models discussed above. Furthermore, as the null Nrf2 gene region is introduced into all cells in the knockout mouse, the T cell and DC function may be influenced by the dysfunction of other cell types which have not been examined. It would therefore be beneficial to assess T cell and DC function in an *in vivo* setting using conditional knockout animals, where the Nrf2 gene is genetically removed from specific cell types. This could be achieved by initially generating Nrf2^{flox/flox} mice through the introduction of LoxP sites into the Nrf2 DNA-binding region (Kong et al., 2011). Targeted ablation of Nrf2 in DCs, for example, could then be attained by crossing the Nrf2^{flox/flox} mice with Cre transgenic mice where the Cre recombinase is under the control of the CD11c promoter (Zhang et al., 2013). Dendritic cell and T cell function were examined individually in this thesis. However, it would be more informative to

assess the precise role of Nrf2 in both DCs and T-cells function during *in vivo* T-cell DC interactions. Utilisation of adoptive transfer methodologies in which the Nrf2 deficient DCs and T cells could be injected into a recipient mouse would help address such questions.

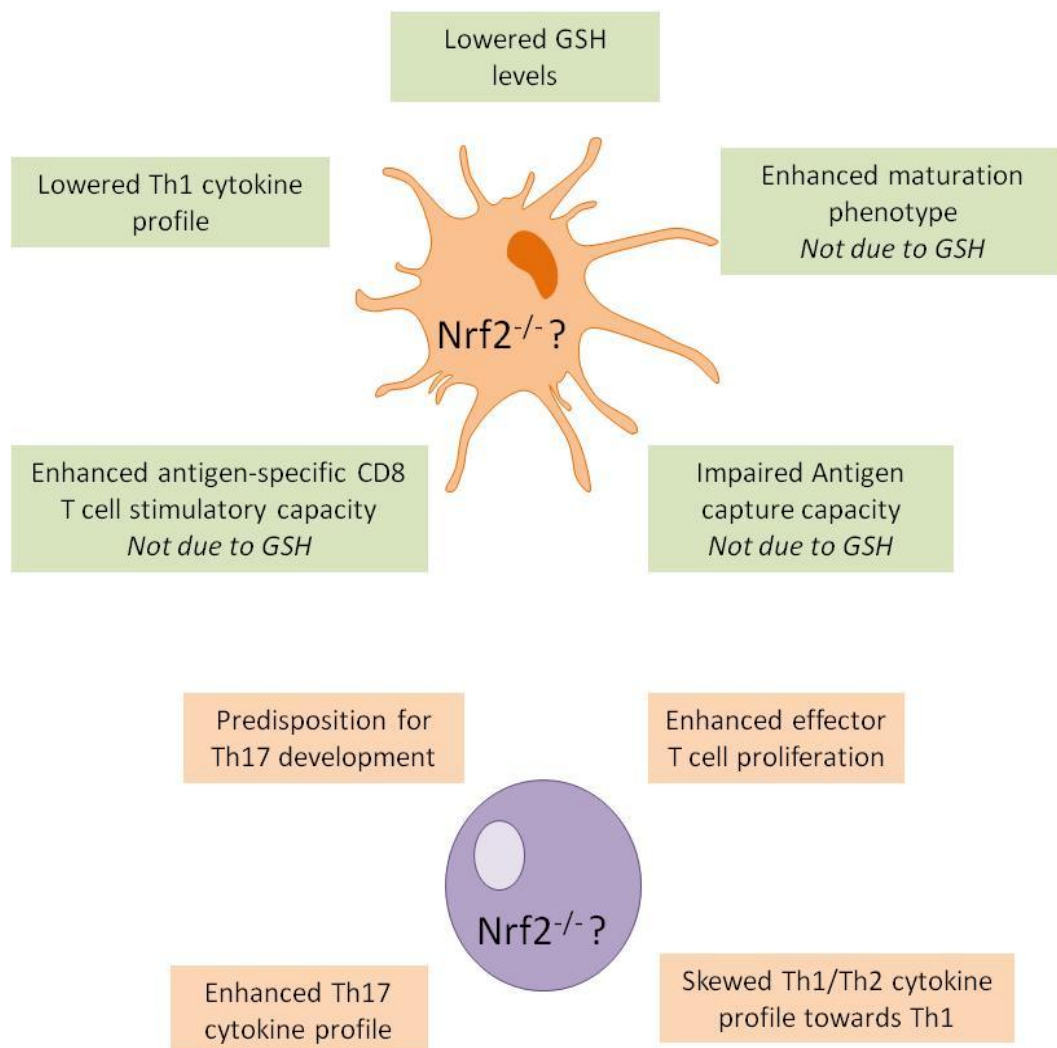


Figure 6.1 Summary of findings on the impact of loss of Nrf2 on murine dendritic cell and T cell immune function.

Extensive research has been carried out on delineating the cytoprotective and drug-detoxifying role of Nrf2 in the liver, which as the primary drug-metabolising organ, is subjected to an array of xenobiotics and their respective metabolites. Individuals

vary in their responses to xenobiotic exposure from adaptation to severe ADRs. However, it is unknown whether this human disparity in drug response is a consequence of inter-individual variation in the Nrf2 adaptive defence system to xenobiotic stress. In light of this, the work presented in the final chapter aimed to firstly decipher whether variation in the Nrf2 adaptive system was present within individuals in response to a known Nrf2 probe. To address the latter, basal and induced Nrf2 protein levels, and downstream NQO1 expression were measured in human T cell blasts, in response to increasing concentrations of the Nrf2 inducing drug CDDO-Me. Blood-derived immune cells represent an accessible source of physiologically relevant cells, thus proved a suitable human *ex vivo* model to examine inter-individual variation in the nrf2 adaptive defence system. It was shown that Nrf2 could be detected in the nuclear fractions of human T cells. Furthermore, upon examination of various donor-derived T cells, it was concluded that humans vary in their Nrf2 response to CDDO-Me, with respect to nuclear Nrf2 and NQO1 mRNA expression. The next step would be to investigate whether inter-individual variation in the Nrf2 response exists in humans in response to an xenobiotic e.g. acetaminophen, and subsequently correlate Nrf2 inter-individual variability with individual susceptibility to drug toxicity. This may attempt to utilise Nrf2 expression in the blood as a suitable biomarker for the detection of potential drug toxicity in inaccessible organs such as the liver. However, as there are various dietary constituents and forms of environmental exposure which are known to induce Nrf2 expression in humans (as discussed in chapter 5) (Wu et al., 2010; Boettler et al., 2011a; Harvey et al., 2011; Messier et al., 2013), the use of Nrf2 as a biomarker for predicting drug toxicity in practice would prove extremely difficult.

A detailed knowledge of potentially altered function of Nrf2 in disease states will open up the possibility of Nrf2 being a viable therapeutic target in such disease settings. Nrf2 polymorphisms have been identified in a variety of immune-mediated human diseases, which often correlate with increased risk of disease severity as seen in acute lung injury (Marzec et al., 2007), nephritis associated in female childhood onset in SLE (Cordova et al., 2010), *Helicobacter pylori*-induced gastritis (Arisawa et al., 2007) and ulcerative colitis (Arisawa et al., 2008). Moreover,

combined maternal Nrf2 single nucleotide polymorphisms (SNP) and acetaminophen exposure throughout pregnancy, has been shown to correlate with enhanced risk of childhood asthma (Shaheen et al., 2010). Other polymorphisms have been identified in SLE and COPD, but correlation with the disease is yet to be determined (Yamamoto et al., 2004). In spite of this, diminished Nrf2 expression and subsequent downregulation of Nrf2 governed antioxidant gene expression e.g. HO-1 and NQO1 have been observed in alveolar macrophages and lungs of COPD patients (Malhotra et al., 2008; Suzuki et al., 2008).

Senescence is undoubtedly correlated with a decline in the functioning of all major organs including the immune system thus increasing susceptibility to an array of disorders including viral and bacterial infections, cardiovascular, pulmonary and neurodegenerative diseases, diabetes and cancer (Ponnappan and Ponnappan, 2011). Immunosenescence is highly associated with chronic levels of oxidative stress. This results in immune cell dysfunction, decreased immunosurveillance, enhanced inflammation and autoimmunity (Ponnappan and Ponnappan, 2011). It has also been suggested that there is an age-related decline in nrf2-mediated antioxidant and detoxification processes to nullify this increased oxidative challenge, ultimately resulting in disease (Kim et al., 2007; Suzuki et al., 2008). In support of this, Nrf2 expression is decreased in human alveolar macrophages derived from older smokers in comparison to younger smokers (Suzuki et al., 2008). Reduced Nrf2 protein expression was also observed between young and aged mice exposed to cigarette smoke (Suzuki et al., 2008). Furthermore, increased nrf2 expression has been observed in PBMCs derived from asthmatic children (Fitzpatrick et al., 2011); whereas decreases in Nrf2 expression has been shown in adult asthmatic alveolar macrophages (Dworski et al., 2011). Nrf2 activation has proven to restore Th1 immunity in aged mice, highlighting a potential role for targeting Nrf2 in the treatment of age-related disorders (Kim et al., 2008).

In reference to the role of Nrf2 in SLE, striking comparisons between the Nrf2 deficient mouse and the autoimmune disease can be made. Nrf2 deficient mice develop complex disease manifestations characteristic of SLE including female dominance, multiorgan inflammation, presence of nuclear auto-antigens and

immune complex deposition in blood vessels and glomerular nephritis (Ma et al., 2006). The present thesis has demonstrated that loss of Nrf2 in mice resulted in enhanced DC co-stimulatory receptor expression, reduced phagocytosis of apoptotic cells, increased DC mediated T cell proliferation in response to self-antigen and increased predisposition of Th17 development, which have collectively been observed in SLE (Berkun et al., 2008; Shah et al., 2010). This highlights a critical role for Nrf2 in this autoimmune disease. However, it has yet to be established whether Nrf2 expression is altered in the immune cells of SLE patients. The human T cell *ex vivo* model presented in this thesis could be used as an appropriate tool to examine basal and inducible levels of Nrf2, and downstream gene expression in SLE patients in comparison to healthy individuals. Hypothetically, if Nrf2 levels were found to be compromised in SLE patients, it would be appropriate to treat SLE patient-derived immune cells with CDDO-Me to assess whether Nrf2 activation enhances their function. CDDO-Me had initially showed great promise as a potential therapeutic for the treatment of chronic type 2 diabetic nephropathy (Pergola et al., 2011). However, it was terminated at phase III clinical trial stage as a consequence of the induction of adverse side effects (Zoja et al., 2013). However, this does not rule out the possibility of CDDO-Me potential treatment of SLE. This merits intense investigations in the future.

The findings from this PhD work could form the basis for several lines of future research. In particular, the role of Nrf2 in DC-mediated immune tolerance and its potential for pharmacological modulation to achieve tolerance in allergic and autoimmune conditions could be investigated. Another strand of future investigation can focus on defining the mechanisms by which Nrf2 influences Th17 development and function. Finally, the hypothesis that Nrf2 function in immune cells is dysregulated in human diseases such as SLE requires testing.

In conclusion, my work has provided a body of scientific evidence that supports the case for a pivotal role of Nrf2 in immune cell development and function. It is hoped that future studies will be able to build on and exploit my findings in order to devise Nrf2-targeted therapies for immune-mediated disease.

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